Medical Virology: From Pathogenesis to Disease Control
Series Editor: Shailendra K. Saxena

Aditya Arya
Mairaj Ahmed Ansari *Editors*

Molecular Diagnostics for Viral Diseases

Challenges and Emerging Concepts

Springer

Medical Virology: From Pathogenesis to Disease Control

Series Editor

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Lucknow, India

This book series reviews the recent advancement in the field of medical virology including molecular epidemiology, diagnostics and therapeutic strategies for various viral infections. The individual books in this series provide a comprehensive overview of infectious diseases that are caused by emerging and re-emerging viruses including their mode of infections, immunopathology, diagnosis, treatment, epidemiology, and etiology. It also discusses the clinical recommendations in the management of infectious diseases focusing on the current practices, recent advances in diagnostic approaches and therapeutic strategies. The books also discuss progress and challenges in the development of viral vaccines and discuss the application of viruses in the translational research and human healthcare.

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Editors

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Preface

Molecular diagnostics for viral diseases is deeply rooted in the remarkable history of Molecular Biology and Virology. In 1869, Johann Friedrich Miescher first identified deoxyribonucleic acid (DNA), laying the foundation for understanding genetic material. Over the decades, groundbreaking discoveries such as Oswald Avery, McCarty, and Colin MacLeod's revelation in 1944 that DNA is the transforming principle, and Erwin Chargaff's demonstration of species-specific DNA composition in 1949, helped shape modern genetics. Linus Pauling's characterization of sickle cell anemia in the same year illustrated the concept of molecular diseases. The 1953 double helical model of DNA by Watson and Crick became a milestone, propelling molecular biology forward. Subsequent innovations, including Arthur Kornberg's isolation of DNA polymerases (1958), Marshall Nirenberg's discovery of the genetic code (1961), and the advent of hybridization techniques in the 1960s, created a robust platform for molecular diagnostics. By the 1970s, the isolation of restriction enzymes (Hamilton Smith 1970) and the first recombinant DNA molecule (Paul Berg 1972) marked the dawn of genetic engineering. The revolutionary polymerase chain reaction (PCR), developed by Kary Mullis in 1983, transformed the field, enabling the rapid amplification of genetic material and establishing the foundation for modern diagnostic tools. The emergence of real-time PCR (Higuchi et al. 1992) and DNA microarrays (Derisi et al. 1996) further refined the accuracy and speed of molecular diagnostics, ultimately leading to the first draft of the human genome sequence in 2001.

The need for robust molecular diagnostic techniques became more evident than ever with the outbreak of the COVID-19 pandemic. The SARS-CoV-2 crisis underscored the critical importance of rapid and reliable viral detection methods to mitigate global health emergencies. Traditional diagnostic approaches proved insufficient in the face of the pandemic's scale, necessitating an unprecedented reliance on RT-PCR, CRISPR-based detection, and next-generation sequencing (NGS) techniques. Beyond SARS-CoV-2, the global community continues to grapple with emerging and re-emerging viral threats such as Ebola, Zika, H1N1, and Nipah, making the evolution of molecular diagnostics a crucial area of study. Despite significant advancements, challenges remain, including the need for greater sensitivity,

accessibility, and speed in viral detection. This book aims to bridge these gaps by addressing both the challenges and the emerging concepts in molecular diagnostics, equipping researchers, clinicians, and policymakers with the knowledge to navigate future outbreaks effectively.

As experts with over a decade of experience in molecular diagnosis of viral infections, we have curated this book, with the help of eminent researchers in the domain, to serve as an authoritative resource for students, researchers, and healthcare professionals. The present book highlights the critical role of genomic and proteomic technologies in identifying, characterizing, and combating viral diseases. We explore cutting-edge innovations such as digital PCR, point-of-care diagnostics, and emerging diagnostic approaches that promise to reshape the landscape of virology. In an era of increasing zoonotic spillovers and global travel, preparedness is paramount. We hope that this book will not only serve as an academic reference but also as a catalyst for further research and innovation in molecular diagnostics, ultimately aiding in the global fight against viral diseases. We extend our gratitude to the pioneers whose contributions have shaped this field and to our readers, who will continue this vital work into the future.

New Delhi, Delhi, India

Aditya Arya
Mairaj Ahmed Ansari

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Chapter 1
Introduction to Molecular Diagnostics and Viral Diseases

Rakesh Kaushik, Shilpa Mahajan, Gopal Dass, and Manish K. Chatli

Abstract The twenty-first century has been marked by numerous viral disease outbreaks, including the devastating COVID-19 pandemic, alongside SARS, H1N1, MERS, Ebola, and Zika, each causing widespread illness and mortality. Historically, identifying novel pathogens was a lengthy process, often taking years or decades. However, advancements in molecular diagnostics have significantly shortened this timeline, enabling the rapid identification of pathogens within weeks. Molecular diagnostics has emerged as a pivotal tool in the identification and management of viral diseases, significantly enhancing the accuracy and efficiency of disease detection. This chapter begins with an introduction to the principles of molecular diagnostics, elucidating its importance in addressing the growing burden of viral infections globally and then explores the escalating trends of viral infections, emphasizing their socio-economic implications on public health systems and economies. It critically examines the limitations of conventional diagnostic methods, highlighting the necessity for molecular approaches that offer enhanced sensitivity and specificity. Various strategies for molecular diagnosis, including polymerase chain reaction (PCR) and next-generation sequencing (NGS), are discussed, along with current market trends that indicate a shift toward more integrated and accessible diagnostic solutions.

Keywords Molecular diagnosis · COVID-19 · PCR · Viral infections · NGS

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1.1 Introduction

The twenty-first century has witnessed a series of significant infectious disease outbreaks, with the COVID-19 pandemic particularly devastating global health and economies. Other notable events include the 2003 outbreak of severe acute respiratory syndrome (SARS), the 2009 H1N1 influenza pandemic, the 2012 Middle East respiratory syndrome (MERS) outbreak, the Ebola epidemic in West Africa from 2013 to 2016, and the Zika virus outbreak in 2015. Each of these outbreaks resulted in substantial morbidity and mortality, transcending national borders and affecting multiple countries (Baker et al. 2022). Reflecting on historical pandemics caused by novel pathogens, the process of pathogen identification and characterization traditionally spanned years, decades, or even centuries. However, with advancements in molecular techniques, the timeline for identifying new pathogens has been significantly reduced to mere weeks or months. A prominent example is the rapid detection of SARS-CoV, which was identified within weeks of its initial reported cases. The integration of advanced technologies and molecular methods is becoming increasingly indispensable in clinical microbiology laboratories for detecting, identifying, and characterizing infectious agents. The emergence and re-emergence of pathogens continue to pose significant challenges for diagnosis, treatment, and public health surveillance. Molecular diagnostics is a rapidly advancing field that utilizes techniques from molecular biology to detect and analyze genetic material from pathogens, enabling precise identification of diseases. In the context of viral diseases, these techniques play a crucial role in diagnosing infections, monitoring disease progression, and guiding treatment strategies. Methods such as polymerase chain reaction (PCR), next-generation sequencing (NGS), and real-time PCR allow for the rapid detection of viral RNA or DNA, even from small sample volumes. This technology is essential for early diagnosis, outbreak control, and personalized treatment in the management of emerging and re-emerging viral diseases. Let us have a quick glimpse of molecular diagnosis per se, as a field of biology and approaches used in the domain in following section.

Molecular diagnosis integrates molecular biology techniques with human genetics and medical knowledge to provide insights into therapeutic monitoring and personalized medicine. Although various authors have given varied definitions of "molecular diagnosis" in their literary works, yet, widely accepted meaning of molecular diagnosis encompasses a group of techniques that concentrate on finding patterns and changes in DNA and RNA, which are crucial for the identification, categorization, and prediction of diseases. However, from a wider standpoint, molecular diagnostic procedures encompass *all techniques for clinical condition diagnosis that depend on the identification of biomarkers, especially important biomolecules like proteins and nucleic acids*. Definition from Yale, considers *only nucleic acids as target molecules in molecular diagnosis* while national cancer institute (NCI-NIH) considers *techniques based on proteins as well as nucleic acids in the definition.* Even though the word "diagnosis" is frequently used in this context, these techniques do not always center on illness diagnosis. Molecular

diagnostic techniques can occasionally be applied to the identification of precision therapeutic approaches, genetic predisposition, susceptibility, and prognosis for a variety of diseases. The field of molecular diagnosis spans across various areas such as molecular oncology, epigenetics, toxicology, immunotherapy, and clinical pathology, among others, reflecting its broad scope in human healthcare. The journey of molecular diagnostics began long before modern technology, tracing back to ancient speculation about the transmissible nature of hereditary material. Hippocrates was one of the earliest to hypothesize about the role of genetic material in inheritance, which later became more defined through Gregor Mendel's work on inheritance patterns in pea plants in 1866. Key milestones such as Friedrich Miescher's discovery of nuclei in pus cells and Albrecht Kossel's isolation of nucleic acids in 1878 laid the foundation for research into genetic material. The early twentieth century marked a significant shift when the clinical approach to understanding disease inheritance was revolutionized by studies on recessive disease patterns, especially in patients with alkaptonuria. By the mid-century, scientific breakthroughs, including Oswald Avery's transforming principle, paved the way for understanding genetic transformation, followed by Watson and Crick's elucidation of the DNA structure in 1953. These discoveries provided the conceptual basis for the molecular diagnosis tools that would soon follow.

The technological explosion in molecular diagnostics came with the invention of PCR by Kary Mullis in 1985, a technique that revolutionized the ability to amplify DNA sequences. This was built on earlier breakthroughs such as the discovery of Taq Polymerase in 1976, which allowed for high-temperature DNA replication. Frederick Sanger's sequencing method, which introduced chain-terminating dideoxynucleosides, was yet another significant advancement in molecular diagnosis and remains foundation stone of automated first-generation sequencing technologies. By the early 1990s, molecular diagnostic tools, particularly PCR, found widespread application in detecting genetic disorders and infections like HIV and hepatitis. The development of next-generation sequencing (NGS) in the 2000s further accelerated the field by enabling faster and more comprehensive sequencing of entire genomes, laying the groundwork for personalized medicine (Fig. 1.1). While newer methods such as DNA sequencing emerged, more methods for a better point of care solution evolved. One such invention was loop mediated isothermal DNA amplification method or LAMP in short. Nevertheless, innovations in metagenomics and fluorescence in situ hybridization (FISH) allowed for the detection of pathogens and cancer genes with unprecedented precision. By 2007, sequencing techniques, including pyrosequencing and ion semiconductor sequencing, were further refined, allowing for even more accurate detection of genetic material. Additionally, single-molecule real-time sequencing (SMRT) and DNA nanoball sequencing added new layers of depth to molecular diagnostics.

The evolution of molecular biology, accelerated by bioinformatics, has brought about continuous advancements in diagnosing and understanding diseases, making molecular diagnostics an essential component of modern healthcare. Along the same period when, PCR got attention in the field of molecular diagnosis by achieving nucleic acid amplification, efforts of Georges Köhler and César Milstein in early

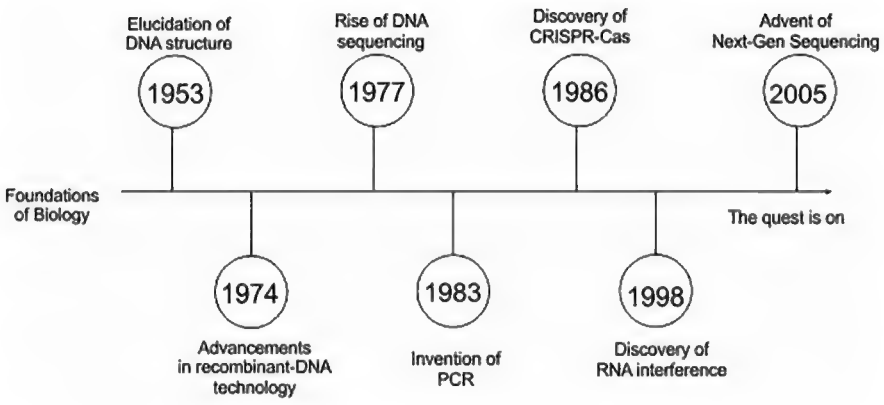

Fig. 1.1 Seminal discoveries that laid the path of molecular diagnostics

1980s added another dimension to the molecular diagnosis using proteins. Their discovery laid the foundations of a highly sensitive and specific detection of proteins using monoclonal antibodies. A wide variety of assays and point of care (POC) molecular diagnosis tools soon flooded the commercial space. Furthermore, several other classical approaches such as immunoblotting and mass spectrometry which were widely accepted in research, have begun to step toward the field of molecular diagnosis. In yet another dimension, the use of short stretches of DNA called aptamers also remained in limelight for a long period of time, but apparently the footprints of the use of technology are not prominent enough. The field continues to grow, with innovations such as aptamer-based detection and molecular imprinting technologies offering new frontiers for detecting pathogens, genetic mutations, and biomarkers associated with a range of diseases.

Classifying molecular diagnostic methods is crucial for understanding the diverse landscape of techniques available for disease detection, monitoring, and treatment. While writing this book, we were not able to obtain any comprehensive framework classification schema, hence we outlined a basic purview, based on various available approaches at this time. A systematic classification not only facilitates the identification of suitable methodologies for specific clinical applications but also enhances communication among healthcare professionals, researchers, and regulatory bodies. By organizing molecular diagnostics into categories based on factors such as the target of analysis (DNA, RNA, proteins), technological platforms (PCR, next-generation sequencing, microarrays), end user (laboratory professionals, personal use, or point of care devices for health workers), or application fields (oncology, infectious diseases, genetic disorders), we can better assess the strengths and limitations of each method. Some of the broad categories have been elaborated later and illustrated in Fig. 1.2.

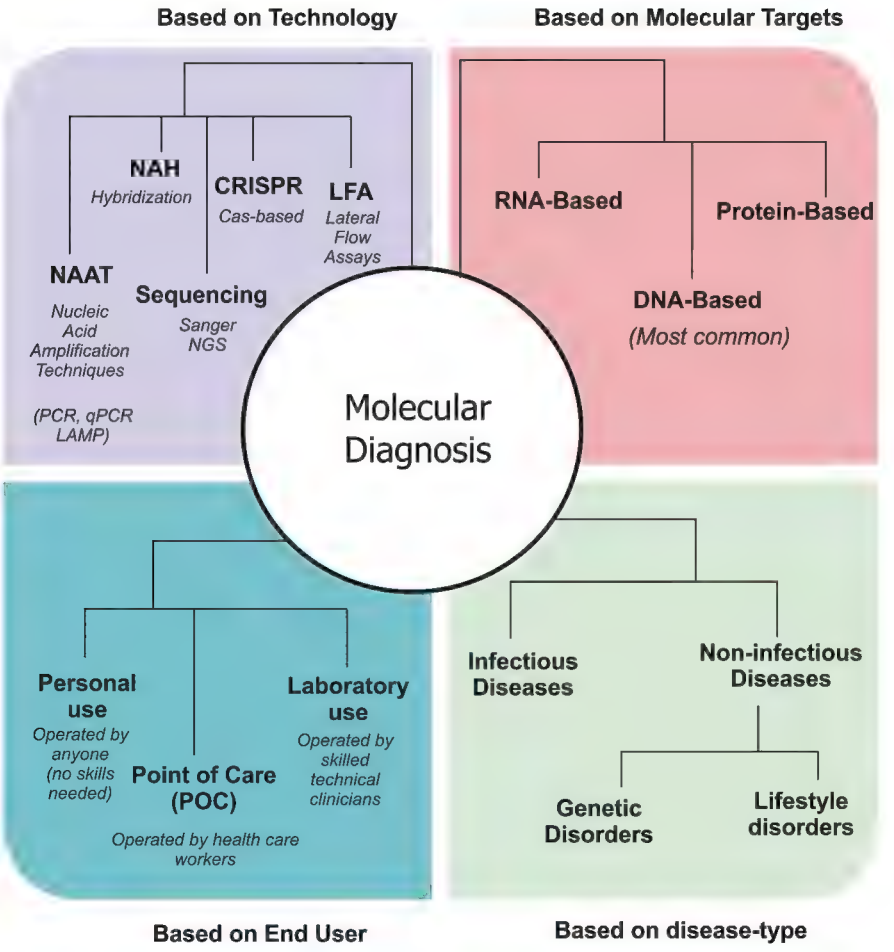

Fig. 1.2 A general scheme of classification of various molecular diagnostic approaches

1.1.1 Based on Target Biomolecule

One of the most fundamental classifications is based on the type of molecule identified in the diagnostic process. DNA-based methods focus on detecting genetic material, typically using techniques such as polymerase chain reaction (PCR) and next-generation sequencing (NGS) to analyze mutations, polymorphisms, and pathogen DNA in a sample. These methods are widely used for detecting genetic disorders, infectious diseases, and cancer-related genetic changes. RNA-based diagnostics, on the other hand, detect and analyze RNA molecules, which can provide insights into gene expression patterns, viral infections, and transcriptomic changes. Techniques such as reverse transcription PCR (RT-PCR) and RNA sequencing (RNA-Seq) are commonly used for detecting RNA viruses, gene

expression alterations in cancer, and inherited genetic conditions involving splicing defects. Protein-based diagnostics focus on identifying and quantifying proteins and peptides, often through techniques such as enzyme-linked immunosorbent assay (ELISA), Western blotting, and mass spectrometry. These methods are particularly valuable for detecting biomarkers of diseases, monitoring immune responses, and identifying bacterial toxins or viral proteins in infectious diseases.

1.1.2 Based on Technology

Another significant classification of molecular diagnostics is based on the technical approach utilized in the detection and analysis process. Nucleic acid amplification-based techniques (NAATs) include methods such as PCR, loop-mediated isothermal amplification (LAMP), and rolling circle amplification (RCA), all of which amplify target DNA or RNA sequences to enable sensitive detection. These methods are widely used for detecting infectious pathogens, genetic mutations, and oncogenes. Nucleic acid hybridization-based techniques rely on the principle of complementary base pairing to detect specific sequences and include methods such as fluorescence in situ hybridization (FISH) and microarrays, which are commonly used for detecting chromosomal abnormalities and gene expression patterns. Lateral flow assays (LFA), including point-of-care (POC) diagnostic tools, offer rapid and easy-to-use molecular detection methods often employed in home testing and field settings. These methods include pregnancy tests, rapid antigen tests for infectious diseases, and portable nucleic acid-based detection systems. CRISPR-based (Clustered Regularly Interspaced Short Palindromic Repeats) diagnostics have recently emerged as a powerful molecular diagnostic tool, leveraging CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats) enzymes to detect nucleic acids with high specificity and sensitivity. Methods such as specific high-sensitivity enzymatic reporter UnLOCKing (SHERLOCK) and DNA endonuclease-targeted (CRISPR) Clustered Regularly Interspaced Short Palindromic Repeats trans reporter (DETECTR) have been developed for detecting viral and bacterial infections, genetic diseases, and even cancer biomarkers with rapid, cost-effective workflows.

1.1.3 Based on End-User

Molecular diagnostic methods can also be classified based on the end-user and complexity of the testing process. *Point-of-care (POC)* diagnostics are designed for rapid, on-site testing without requiring extensive laboratory infrastructure. These tests are commonly used in clinical settings, home testing, and low-resource environments to provide quick results for conditions such as COVID-19, influenza, and sexually transmitted infections. Most often the POC are meant for the operation by healthcare workers and in some cases for the personal use as well.

Laboratory-based molecular diagnostics require specialized equipment, trained personnel, and controlled environments for high-precision and high-throughput testing. Techniques such as conventional PCR, quantitative PCR (qPCR), spectrometry, and NGS fall into this category and are typically used in centralized diagnostic laboratories for comprehensive disease analysis, genetic screening, and pathogen surveillance. *Personal-use molecular diagnostics* have gained popularity with the development of home testing kits for genetic predisposition analysis, ancestry tracing, and monitoring health conditions such as blood glucose levels and cholesterol. These tests empower individuals to monitor their health and make informed decisions without the need for direct medical intervention, though confirmatory laboratory testing is often recommended for clinical decision-making.

1.1.4 On the Basis of Type of Disease

Another way to classify molecular diagnostics is based on the type of disease being diagnosed. *Molecular diagnosis for infectious diseases* is one of the most widespread applications, utilizing PCR, NAATs, and antigen/antibody detection methods to identify bacterial, viral, fungal, and parasitic infections. This category includes tests for diseases such as tuberculosis, HIV, hepatitis, COVID-19, and malaria, where rapid and precise detection of pathogens is crucial for treatment and containment. *Molecular diagnosis of genetic diseases* focuses on detecting hereditary conditions, chromosomal abnormalities, and single-gene disorders using techniques such as whole genome sequencing (WGS), whole exome sequencing (WES), and gene-specific PCR. These methods help identify conditions like cystic fibrosis, sickle cell anemia, and hereditary cancers, allowing for early diagnosis, genetic counselling, and personalized treatment approaches. *Molecular diagnosis of lifestyle diseases* involves the identification of biomarkers related to conditions such as diabetes, cardiovascular diseases, and cancer. Advanced molecular techniques such as liquid biopsy, DNA methylation analysis, and proteomics are used to detect disease-related mutations, metabolic changes, and early signs of malignancies, providing valuable insights into disease prevention and management. In this book, our main focus is to discuss viral diseases and hence the discussion on diagnosis of other diseases through molecular diagnosis can be explored elsewhere.

1.2 Global Trends of Viral Infections and Their Socio-Economic Impact

For a novel pathogen to threaten human populations, three key conditions must be met: first, there must be contact between humans and the animal reservoir; second, the pathogen must develop the ability for sustained human-to-human transmission; and third, this transmission should facilitate geographical spread beyond the initial

spillover zone (Lloyd-Smith et al.). Recent global changes, including urbanization, climate change, and increased global travel, have amplified the potential for novel pathogens to emerge and spread. For instance, air travel enabled the rapid spread of the SARS coronavirus to 27 countries before containment measures were implemented (Guan et al. 2004).

The rise in air travel, trade, and urbanization has accelerated the movement of people and goods, creating opportunities for infectious diseases to spread. Reports from the World Health Organization (WHO) highlight the ongoing public health challenges related to infectious diseases, particularly as intensive agricultural practices, such as high-density farming, foster conditions that favor pathogen circulation among livestock (Pitzer et al. 2016). This environment not only increases the risk of new livestock pathogens emerging (Jones et al. 2013) but also the possibility of antibiotic-resistant bacteria developing due to widespread antibiotic use in agriculture (Van Boeckel et al. 2020). Global connectivity influences infectious disease risks through three main forms: international travel, human migration, and the international trade of animals and plants. Increased air travel facilitates the rapid spread of pathogens, as evidenced by the SARS outbreak, where a single infected traveler introduced the virus to multiple countries. Patterns of migration driven by economic and environmental factors promote closer interactions between diverse populations, particularly in urban areas. Additionally, global trade can introduce pathogens into new regions, while agricultural practices heighten the risk of zoonotic diseases (Brashares et al. 2004).

The technological advances of the late twentieth and early twenty-first centuries have transformed global travel, with the number of airline passengers doubling from just under two billion in 2000 to over four billion in 2019. This increase in global mobility has heightened the risk of emerging pathogens and suggests that pandemics, like COVID-19, may become more frequent. The unique biological characteristics of SARS-CoV-2, such as asymptomatic and presymptomatic spread, made it particularly challenging to control (Laxminarayan et al. 2013). Urbanization has further complicated these dynamics. As human populations expand into previously unoccupied regions, patterns of contact with wildlife increase, raising the risk of zoonotic spillover events. Factors such as population growth, agricultural expansion, and increased wealth contribute to greater habitat destruction and closer contact between wild and domestic animals, which enhances the likelihood of disease transmission (Aw et al. 2007; Nikolich-Žugich 2018). The decline in immune function associated with aging populations may also increase susceptibility to emerging pathogens.

Antibiotic resistance is a growing global concern, resulting from selective pressure due to widespread drug use. This issue is not limited to bacterial infections; similar resistance has been observed in malaria, where antimalarial resistance has rapidly spread following its emergence (Talisuna et al. 2004). The convergence of aging populations, declining immune defences, and increasing drug resistance points to a future where both new and existing pathogens pose complex threats to global health. Demographic changes, particularly urbanization, can significantly influence pathogen transmission dynamics. As urban populations grow denser, the

risk of disease spread increases due to closer human contact. Climate change also plays a critical role, as shifting environmental conditions can alter species ranges, leading to new interactions between wildlife, humans, and domestic animals (Carlson et al. 2020). Such novel interactions create opportunities for zoonotic diseases to emerge.

Greater connectivity facilitates genetic exchange between populations, accelerating pathogen evolution and potentially eroding host resistance (Zhan et al. 2015). The phenomenon of spillover followed by spillback—where pathogens transfer from animals to humans and back—can amplify pathogen evolution, as seen in the transmission of SARS-CoV-2 between humans and mink (Oude Munnink et al. 2021). Moreover, increased connectivity raises the likelihood of novel vectors spreading diseases globally, exemplified by the global spread of malaria through mosquito vectors (Molina-Cruz et al. 2016). The homogenization of crops and livestock further increases disease susceptibility, as seen in the devastation of the American chestnut tree by chestnut blight (Rigling and Prospero 2018). Changes in healthcare strategies, such as vaccination, can also influence pathogen selection pressures, driving the evolution of virulence. As life expectancy rises and deaths from infectious diseases decline, the primary threat to global health is shifting toward emerging infections. Key drivers of this risk include climate change, rapid urbanization, and changing land-use patterns. However, potential stabilizing factors, such as enhanced health surveillance and public health preparedness, may mitigate some of these risks (Antràs 2020, Rachel E. Baker et al. 2022). Industries play crucial roles in shaping economic and social development worldwide, contributing significantly to gross domestic product (GDP) and employment. In 2021, the service sector accounted for 65.7% of global GDP and 51% of total employment, highlighting its importance in economic growth and technological advancement. The manufacturing sector contributed 28.3% to world GDP and provided 23% of global employment. These sectors are critical in addressing public health challenges as they evolve in response to emerging threats (The World Bank).

Over the past decades, industrialization and economic growth, as evidenced by increases in per capita GDP, have significantly improved wages and household incomes. This economic growth has also driven progress in nutrition, housing, sanitation, medical care, and literacy (Pamuk and van Zanden 2010; Costa and Steckel 1997). These developments have generally enhanced living standards and overall health and welfare across many regions of the world.

However, despite these advancements facilitated by modernization and public health measures, the global community continues to grapple with the challenges posed by both emerging and endemic infectious diseases. These outbreaks not only burden global health systems but also have extensive socioeconomic consequences, disrupting economies, livelihoods, and communities. The persistence of infectious diseases—often intensified by factors such as climate change, urbanization, and globalization—demonstrates that economic and social progress alone cannot fully mitigate the threat of global health crises (Smith et al. 2019). Infectious disease outbreaks of varying scale, including epidemics and pandemics, have had widespread impacts. The COVID-19 pandemic, which the World Health Organization

(WHO) declared a global emergency on January 30, 2020, significantly affected primary, secondary, and tertiary sectors of the world's largest economies (Nicola et al. 2020). These effects included rising healthcare costs, job losses, macroeconomic instability, and the decline of micro, small, and medium enterprises (MSMEs) and informal sectors (Nicola et al. 2020; Rasul et al. 2021). Similarly, previous health disasters, such as the severe acute respiratory syndrome (SARS) pandemic in 2003, caused a global economic loss of approximately USD 40 billion due to its impact on industries like hospitality, transport, and multinational enterprises, including the oil sector (Smith et al. 2019). The influenza A (H1N1) pandemic of 2009–2010 also resulted in severe economic recessions and significant declines in stock market values (Shang et al. 2021).

The global molecular diagnostics market was valued at approximately USD 15.20 billion in 2023 and is projected to grow at a compound annual growth rate (CAGR) of 4.5% from 2024 to 2030 (Molecular Diagnostics Market Size and Share Report 2030 Report ID: 978-1-68038-086-6). Besides the developed economies, emerging economies are increasingly adopting molecular diagnostic technologies, driven by improvements in healthcare infrastructure and rising awareness of infectious diseases. Countries like India and Brazil are making substantial investments in diagnostics, with initiatives aimed at enhancing access to advanced testing. Moreover, the Indian market for molecular diagnostics is expected to reach USD 671.8 million by 2030, driven by government initiatives and private sector investments in Molecular Diagnostics technologies and services. Figure 1.3 illustrates the overview of market trend in the molecular diagnosis.

Infectious diseases, both large-scale and smaller outbreaks, impose socioeconomic costs. For example, seasonal influenza leads to absenteeism, reduced productivity, and impaired performance (Szucs 1999). Norovirus outbreaks in semi-enclosed environments have caused losses of up to USD 2 billion in the United States alone

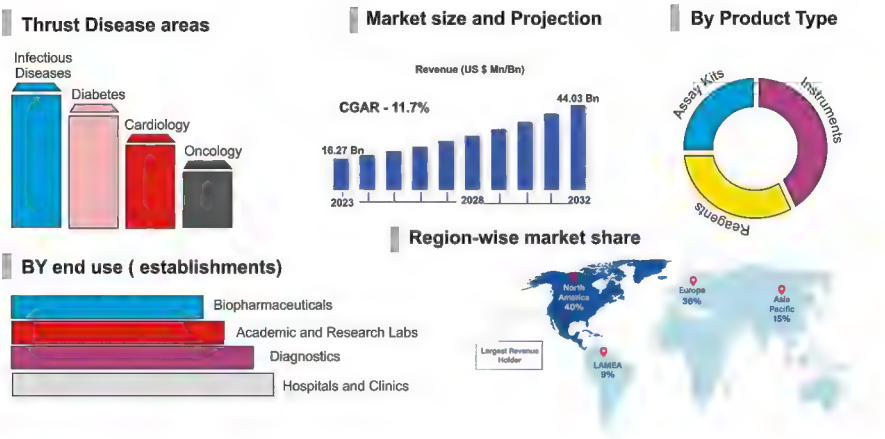

Fig. 1.3 Market trends of molecular diagnostics market (insights based on reports from Fairfield Market Research, Zion Market Research and vision research reports)

due to lost productivity and healthcare expenses. Thus, while industrialization has offered numerous benefits, it remains critical to address the complex health challenges that persist worldwide (Centers for Disease Control and Prevention [CDC] 2023). Endemic diseases like tuberculosis have severe consequences, particularly for the labor force and local economies. Tuberculosis alone is projected to result in an economic loss of USD 17.5 trillion from 2020 to 2050 across 120 countries (Silva et al. 2021). Evidence shows that airborne pathogens like SARS-CoV-2, SARS-CoV, and the influenza virus are transmitted through airborne routes, resulting in superspreading events that disrupt global economies (Wang et al. 2021a, b). The rising costs associated with infectious diseases highlight the importance of evaluating their socioeconomic impacts across multiple industries. Understanding these effects is essential for assessing disease burdens and informing long-term prevention, preparedness, resource optimization, and prioritization strategies. However, most current studies on the socioeconomic impacts of infectious diseases focus on specific regions or individual diseases, such as COVID-19, influenza, or tuberculosis (Bashir et al. 2020; Lenzen et al. 2020; Silva et al. 2021). Socioeconomic impacts on industries are broad, encompassing both social and economic dimensions such as morbidity and mortality, costs of diagnosis and treatment, productivity loss, employment disruption, financial loss, and interruptions to supply chains (Shang et al. 2021; Lin 2020). Studies report both direct impacts, which occur during disease outbreaks, and indirect impacts, such as subsequent changes resulting from the initial effects (Bhuiya et al. 2021). Direct impacts of airborne and droplet-borne infectious diseases on industries include substantial healthcare costs and public health expenses tied to containment measures such as lockdowns, movement restrictions, and temporary shutdowns (Al Ghunaim et al. 2021). Indirect consequences include productivity losses, societal costs, and broader economic impacts. For example, the COVID-19 pandemic led to a 49% reduction in productivity across industries, along with impaired work quality and reduced employee engagement (Al Ghunaim et al. 2021). Among healthcare workers, there were reports of increased medical errors, doubts about career choices, and serious considerations of leaving the workforce (Haidari et al. 2021; Jiménez Labaig et al. 2021). Significant societal costs were also reported due to employment crises and workforce reductions during the COVID-19 pandemic. Workers across industries faced termination, furloughs, or were placed on paid leave during this period (Fargen et al. 2020; Gashi 2021). Moreover, many companies reduced salaries and work hours, leading to severe staff shortages (Alsharef et al. 2021; Brophy et al. 2021). Similar workforce challenges were seen during the SARS and H1N1 epidemics (Bergeron 2006; Duarte et al. 2017). However, certain industrial sectors, such as chemicals and plastics, experienced employment growth during the COVID-19 pandemic compared to pre-pandemic levels (Webster et al. 2022). The aviation industry, on the other hand, faced job losses predicted to reach 25–30 million by the end of 2020 (Iacus et al. 2020).

In total, global economic losses from the influenza, H1N1, SARS, and COVID-19 outbreaks were estimated at USD 600 billion, USD 360 billion, USD 40 billion, and USD 8.5 trillion, respectively (McKibbin and Lee 2009; Fan et al. 2018). The World

Bank projected that the global economy would contract by 5.2% by the end of 2020 due to COVID-19, marking the worst recession since World War II (World Bank 2020). Poorer countries, already facing economic strain and limited health capacities, experienced the most severe impacts (Rasul et al. 2021). It is clear that infectious diseases can impose severe socioeconomic costs on regional and global industries. To maintain economic growth and stability during pandemics, bold policy measures and innovative mechanisms are essential. Strengthening disease surveillance, prevention, preparedness, and response systems, along with investing in vaccine development and distribution, are critical steps to mitigate the threat of infectious diseases. Public health strategies such as coordinated stay-at-home orders, rapid testing, and transparent communication are vital for managing pandemics. Additionally, digital health innovations, such as telehealth and geographic information systems (GIS) for risk mapping, offer innovative solutions during outbreaks. Further research is needed to understand the long-term socioeconomic impacts of infectious diseases, which will support better preparedness and response strategies for future outbreaks. Collaborative cost-sharing approaches involving public and private stakeholders at local, national, and international levels are crucial for ensuring global health and economic security (Samsudin et al. 2024).

1.3 Limitations of Conventional Diagnosis and Need for Molecular Diagnostics

The rising incidence of infectious diseases poses a significant threat to human health, often leading to critical illnesses that can develop rapidly. To facilitate effective diagnosis and treatment, the adoption of advanced etiological detection techniques is essential. Traditional methods, such as microbial culture, microscopic examination, antigen detection, and nucleic acid testing, are commonly employed but present limitations, including prolonged turnaround times, low sensitivity, and restricted detection scopes, particularly for rare or emerging pathogens. Therefore, there is an urgent need for enhanced pathogen detection tools to improve clinical outcomes (Gu et al. 2023). For over a century, microbiologists have sought faster methods for microbial identification, relying on morphological traits and growth patterns. While techniques such as bacterial fermentation and fungal morphology have been used, many traditional methods lack the sensitivity required for accurate identification. In recent years, advances in molecular biology have revolutionized microbial identification, allowing for precise differentiation of pathogens. Molecular biology has become crucial in biological and biomedical research, significantly impacting clinical diagnostics. Various technologies have emerged, including amplification technologies, blotting techniques, electrophoresis, microarrays, and single nucleotide polymorphism (SNP) analysis, driving growth in the molecular diagnostics field.

Molecular diagnostic tests analyze key biomarkers, such as DNA, RNA, or proteins, to detect diseases, monitor progression, evaluate treatment responses, or predict predispositions. Techniques often involve analyzing DNA sequences or gene expression. Biomarker analyses provide insights into genotypic or phenotypic changes associated with diseases. Furthermore, they play a critical role in drug discovery and patient monitoring. One major advantage of molecular diagnostics is their ability to multiplex, enabling the simultaneous detection of multiple genetic targets. This capability saves time and reduces costs compared to traditional single-analyte immunoassays. HIV detection illustrates the benefits of DNA-based tests in terms of specificity and speed. In the late 1980s, conventional immunoassays detected HIV infection after about 22 days, whereas nucleic acid testing (NAT) reduced this window to just 11 days post-infection. A significant advantage of molecular diagnostics is their ability to amplify genetic material using techniques like the polymerase chain reaction (PCR), which enhances sensitivity and allows for the detection of trace amounts of genetic material, unlike traditional protein-based methods. Consequently, molecular diagnostics have become powerful tools for early and accurate disease detection. The field gained momentum following the successful sequencing of the human genome in 2003. DNA testing has transitioned from research to a dominant platform in clinical medicine, emphasizing its growing importance (Debnath et al. 2010). Clinical microbiology laboratories utilize various techniques for the diagnosis, treatment, and prognosis of infectious diseases. The diagnostic process typically involves direct pathogen detection or indirect detection through immune responses, such as identifying pathogen-specific IgM for acute infections. There is an increasing shift toward molecular techniques due to their enhanced accuracy. Nucleic acid amplification techniques, particularly PCR, are widely used for pathogen detection, with real-time PCR providing faster detection and strain differentiation.

Quantitative PCR is critical for assessing disease progression and treatment efficacy. Other molecular methods, like isothermal amplification, are gaining traction, while microarray technology shows promise despite its high costs. Genotyping techniques to identify pathogen subtypes are essential for risk assessment. Clinicians must appreciate the accuracy and speed of molecular methods while acknowledging their limitations (Kurkela and Brown 2009). The roots of molecular diagnostics trace back to the discovery of the DNA double helix in 1953, leading to advanced technologies for high-throughput genotyping and whole-genome analysis. These advancements enhance our understanding of inherited diseases and highlight the significance of molecular diagnostics in patient care. Numerous genetic tests are performed globally, making laboratories specializing in molecular diagnostics vital to medicine. Current sequencing platforms enable new-born screening for treatable inherited diseases, while future applications may include identifying children at risk for diseases and predicting late-onset risks for preventive care. Pre-emptive genotyping for drug metabolism is crucial for personalized medicine, tailoring treatments to individual genetic profiles. Molecular diagnostics present considerable

growth opportunities by targeting therapeutics with greater precision. The influence of single nucleotide polymorphisms (SNPs) and other markers is increasingly recognized, with expectations that validated markers will continue to expand, enhancing their diagnostic value. Future therapies will ideally be guided by this information with minimal toxicity and effective treatment (Zlotogorski et al. 2002).

The rapid advancements in molecular diagnostics after human genome sequencing have solidified its place in pharmacogenomics and personalized medicine. These methods assess individuals' genetic predisposition to drugs, optimizing dosages, and identifying potential side effects. By ensuring the right drug is administered at the right dose, molecular diagnostics improve treatment efficacy. Compared to traditional microbiology, molecular diagnostics provide faster results and greater sensitivity. Their capabilities are increasingly recognized in addressing global trends in emerging infectious diseases. Recent outbreaks, such as SARS and avian influenza, have highlighted the importance of these technologies in managing public health challenges (Debnath et al. 2010). In conclusion, molecular diagnosis encompasses the detection of pathogenic mutations in DNA and RNA samples, facilitating disease detection, diagnosis, subclassification, prognosis, and therapy monitoring. It utilizes various techniques to identify underlying defects in inherited diseases, enhancing clinicians' diagnostic capabilities. Molecular diagnostics continue to translate discoveries into clinical tests, predicting responses to specific therapies. While over 80% of current molecular tests focus on infectious disease detection, the technologies extend to forensic medicine, paternity testing, tissue typing, oncology, and food safety.

Moreover, molecular diagnostic testing requires small tissue samples, often from biopsies, and can be performed on archival material, enhancing accessibility in clinical practice (Debnath et al. 2010). Molecular detection of microbes with known susceptibility profiles serves as an effective alternative to traditional culture methods, exemplified by the molecular detection of *Bordetella pertussis*, enabling rapid diagnosis and treatment. By leveraging molecular techniques, clinicians can improve patient outcomes through prompt interventions (Buck 1996). Looking ahead, molecular diagnostics is expected to play a vital role in medical practice and public health. Molecular genetic testing will enhance disease detection, monitor drug responses, and identify genetic modifiers. Whole genome sequencing is poised to become the gold standard for assessing DNA variations. Challenges like test selection and cost-effectiveness must be addressed for broader adoption of these techniques (Patrinos et al. 2017). The future of molecular diagnostics is promising, with its transformative potential in personalized medicine and precision healthcare. As research continues to explore the human genome and microbial interactions, molecular diagnostic technologies will enhance our ability to effectively diagnose and manage infectious diseases, ultimately improving patient outcomes and healthcare management.

1.4 Various Strategies for Molecular Diagnosis

Molecular diagnostics, primarily driven by nucleic acid tests (NAT), play a crucial role in detecting DNA or RNA sequences associated with genomic abnormalities or polymorphisms. Over the last two decades, NATs have predominantly relied on hybridization-based approaches, which utilize labeled probes for detection. Certain commercial NATs specifically target ribosomal RNA, which offers high sensitivity due to its abundance in cellular environments. To detect low-abundance RNA or DNA, amplification techniques are employed, categorized into target, probe, or signal amplification methods. The most widely recognized target amplification technique is polymerase chain reaction (PCR), initially developed by Roche. Other methods, including strand displacement amplification (SDA), transcription-mediated amplification (TMA), and nucleic acid sequence-based amplification (NASBA), have also emerged from various companies. PCR has established itself as the gold standard in molecular diagnostics, attributed to its versatility, with Roche commanding approximately 47% of the molecular diagnostics market (Debnath et al. 2010).

Historically, microbiologists have sought faster and more efficient microbial identification techniques, traditionally relying on morphological characteristics and growth patterns. Methods employed in this realm include bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects. While some phenotypic traits—such as isoenzyme profiles, antibiotic susceptibility, and chromatographic analysis of cellular fatty acids—provide sufficient sensitivity for strain characterization (Thurm et al. 1994; Lin et al. 1995), many traditional methods fall short. However, advances in molecular biology have revolutionized microbial identification over the past decade, facilitating genomic analysis and enabling precise differentiation and characterization of microorganisms (Mullis 1987, 1990; Erlich et al. 1991).

Early studies involving DNA hybridization revealed bacterial relatedness and contributed to the development of nucleic acid probe technology, which, along with advancements in plasmid and bacteriophage analysis, has become a powerful tool in epidemiology for tracking infectious disease transmission. Nevertheless, like traditional phenotypic methods, these techniques are limited by their dependence on microbial recovery and growth. The introduction of nucleic acid amplification, particularly PCR, has surmounted these limitations by facilitating microbial detection and characterization without the necessity for microbial growth (Mullis et al. 1987; Persing 1991; Wagar 1996). PCR and other amplification methods have streamlined microbial identification, broadening diagnostic capabilities through techniques such as probe hybridization, fragment analysis, and direct sequencing. Virus detection for diagnosing infections can be achieved through indirect or direct identification methods. Indirect methods involve isolating the virus by introducing it into host cells, utilizing traditional culture methods (Leland et al. 2007) or faster centrifuge-enhanced techniques (Gleaves et al. 1984; Hematian et al. 2016). Detection occurs through observing morphological changes and cytopathic effects (CPEs) or

intracellular staining of viral proteins prior to the manifestation of CPEs. In contrast, direct methods identify the virus from the original source without propagation, employing immunological or molecular techniques. Immunological methods utilize antibodies—whether polyclonal, monoclonal, or recombinant—to detect viral antigens via techniques like blotting or ELISA (Ma et al. 2015; Usuda et al. 2000; Yu et al. 2005). However, these methods can be expensive, require specialized personnel, and often lack the sensitivity of molecular detection (Hema et al. 2021).

Molecular identification of viruses relies on NATs, which involve extracting and purifying pathogen nucleic acids from biological samples such as blood, urine, or saliva, followed by detection using PCR. PCR amplifies a specific genetic sequence unique to the pathogen using polymerase enzymes and thermal cycling. Innovations such as quantitative real-time PCR (qPCR) facilitate the quantification of genetic targets via fluorescent labels (Petralia et al. 2017; Almassian et al. 2013). Despite its maturity in diagnostics, NATs remain time-consuming, costly, and require specialized equipment, which limits their applicability for point-of-care (POC) settings or rapid deployment during pandemics (Wang et al. 2021a, b). Consequently, NATs are evolving toward faster, portable, and integrated POC technologies. The escalating demand for large-scale viral infection diagnostics, coupled with resource limitations in certain infrastructures, has driven the development of integrated, low-cost, and portable platforms for rapid, decentralized analysis. This evolution underscores the necessity for early virus detection to improve diagnostic accuracy. Over time, viral detection methodologies have transitioned from conventional indirect identification—relying on cultural evaluations of viral cytopathic effects in host cells—to more direct methods, such as immunological detection of viral antigens and molecular analyses of viral genomes using NAT. The molecular approach, which encompasses nucleic acid extraction and PCR amplification, offers the most precise and comprehensive diagnostic results. Despite the advancements and increasing accessibility of NAT, it remains confined to specialized laboratories due to complex and lengthy procedures. Significant efforts are underway to integrate NAT into more automated and compact systems by leveraging advancements in solid-phase extraction (SPE) and isothermal PCR methods to facilitate point-of-care genetic testing.

The evolution of conventional NATs for virus detection has been pivotal in achieving necessary miniaturization, integration, and automation. The objective is to streamline nucleic acid analysis—encompassing extraction, amplification, and detection—into a portable, user-friendly solution that can be operated outside of a laboratory setting by non-specialized personnel. Considerable advancements have been made, particularly in improving the purification and isolation steps of the nucleic acid extraction process, as well as enhancing qPCR reactions through optimized probe chemistry and thermal cycling protocols (Sciuto et al. 2021). Molecular techniques have significantly transformed diagnostic virology by enabling direct detection of viral nucleic acids in patient samples (Acharya et al. 2004). Despite the increasing adoption of nucleic acid-based methods, immunoassays continue to play a crucial role in detecting and sero-surveillance of human viral infections.

Immunological techniques identify viral infections by detecting either antiviral antibodies or viral antigens in clinical samples (Zhang et al. 2014; Chen et al. 2015). These methods are extensively employed in the routine clinical diagnosis of viral infections globally, offering advantages such as high sensitivity, specificity, ease of execution, rapid results, and the capacity to test multiple specimens simultaneously (Souf 2016; Lau 2004). Nonetheless, immunological assays have limitations, including susceptibility to interferences from cross-reactive agents with similar or identical epitopes to the viral antigen, potentially resulting in false-positive results (Souf 2016; Schiettecatte 2012). Additionally, endogenous antibodies present in the specimen can interact with antiviral or detection antibodies, leading to false-positive outcomes even in the absence of the viral antigen (Schiettecatte 2012; Emerson 2013).

The nucleic acid-based diagnostic tests are recognized for their high sensitivity and specificity, making them essential for diagnosing and controlling viral infections. These methods enable the detection of viral RNA or DNA, allowing identification of infected individuals prior to an antibody response. This early detection is particularly crucial for vulnerable populations, including the young, elderly, and immunocompromised. Despite the high costs and complex instrumentation associated with these tests, ongoing efforts focus on developing cost-effective, high-quality diagnostic tests, especially for low-income nations (Reta et al. 2020). Currently, no single method fulfils all requirements for virus detection, and the choice between nucleic acid detection and immunoassay methods largely depends on specific assay requirements, testing environments, and urgency of diagnosis (Cassedy 2021).

1.5 Commercial and Technological Prospects

1.5.1 Technological Advancements

The international molecular diagnostics market is characterized by rapid technological advancements. Techniques such as polymerase chain reaction (PCR), next-generation sequencing (NGS), and microarray technologies are becoming increasingly sophisticated, enabling more accurate detection of viral pathogens. For example, the development of multiplex PCR assays allows for the simultaneous detection of multiple viruses, significantly improving diagnostic efficiency. The rise of emerging infectious diseases, highlighted by the COVID-19 pandemic, has accelerated the demand for molecular diagnostic tests. Rapid tests for viruses like SARS-CoV-2 demonstrated the need for quick, reliable diagnostics, prompting increased investment in research and development (R&D). Companies like Roche and Abbott have expanded their portfolios to include point-of-care tests that deliver results in less than an hour.

1.5.2 Increased Demand for Rapid Testing

The COVID-19 pandemic has significantly altered the landscape of molecular diagnostics in many countries. There is a heightened demand for rapid testing, particularly in the context of infectious diseases. This demand has led to the development of various point-of-care molecular diagnostic tests, enabling healthcare providers to make quicker decisions.

1.5.3 Focus on Public Health Initiatives

National health authorities are increasingly focusing on public health initiatives aimed at improving surveillance and control of viral diseases. For instance, the Centers for Disease Control and Prevention (CDC) in the United States has prioritized molecular diagnostics in its response to outbreaks, enhancing laboratory capacity and promoting the use of rapid testing (CDC 2023).

1.5.4 Investment in Research and Development

National governments are recognizing the importance of investing in R&D for molecular diagnostics. For example, the National Institutes of Health (NIH) in the United States has allocated significant funding for projects aimed at developing novel diagnostic tests for viral diseases. These investments are critical for ensuring the availability of cutting-edge diagnostic technologies.

1.5.5 Emerging Startups and Collaborations

The national molecular diagnostics market is witnessing a surge in startups focusing on innovative testing solutions. Collaborations between established companies and emerging startups are fostering a dynamic environment for innovation. For instance, partnerships between technology firms and healthcare organizations aim to integrate artificial intelligence (AI) with molecular diagnostics, enhancing test accuracy and efficiency (Sánchez et al. 2022).

1.5.6 Regulatory Changes

Regulatory agencies worldwide are adapting to the rapid pace of innovation in molecular diagnostics. The U.S. Food and Drug Administration (FDA) has implemented expedited pathways for the approval of diagnostic tests, particularly those

related to infectious diseases (Mullard 2024). Similarly, the European Union's In Vitro Diagnostic Regulation (IVDR) aims to enhance patient safety while fostering innovation in diagnostic technologies.

1.5.7 Personalized Medicine

The trend toward personalized medicine is reshaping the molecular diagnostics landscape. By identifying specific viral strains and mutations, healthcare providers can tailor treatments to individual patients, improving outcomes. This trend is particularly significant in the context of viral diseases such as HIV and hepatitis C, where viral load and genotype can influence treatment decisions.

While the molecular diagnostics market is expanding, challenges remain in regulatory processes and reimbursement frameworks. The approval of new diagnostic tests can be time-consuming and resource-intensive, which may hinder innovation (Mullard 2024). Additionally, reimbursement policies can significantly impact the adoption of new technologies, with tests lacking clear coverage often facing barriers to market entry (Lieberman et al. 2023). Major players in the molecular diagnostics market include Roche Diagnostics, Abbott Laboratories, Thermo Fisher Scientific, and Qiagen. These companies are actively involved in developing innovative testing solutions for viral diseases. For instance, Roche has introduced a range of COVID-19 diagnostic products, while Abbott's ID NOW platform enables rapid detection of various infectious diseases The integration of artificial intelligence and big data analytics is expected to play a crucial role in the future of molecular diagnostics. By leveraging large datasets, healthcare providers can improve diagnostic accuracy, predict disease outbreaks, and personalize treatment plans (Khan et al. 2023). The trend toward point-of-care testing is likely to continue, driven by the demand for *rapid and accessible diagnostics*. This shift will enable healthcare providers to deliver timely interventions, particularly in remote and underserved areas. The increasing prevalence of viral diseases globally calls for a concerted effort to improve access to molecular diagnostics in low- and middle-income countries. Partnerships between governments, non-government organizations (NGOs), and private sectors will be essential in addressing disparities in healthcare access and ensuring that advanced diagnostic technologies reach those in need (WHO 2023).

1.6 Pre-COVID and Post-COVID Changes in Molecular Diagnostics

COVID-19 pandemic has seemingly created a temporal divide in the usage and development of molecular diagnostics of viral diseases and hence may be considered as a valuable reference point for discussion. Molecular diagnostics' history can be traced back to Hippocrates' early speculation about genetic material transfer.

However, the field's modern transformation began with PCR and the development of automated sequencing machines such as Applied Biosystems' AB370, which revolutionized sequencing through capillary electrophoresis (Liu 2011). The early 2000s witnessed further breakthroughs, particularly with the introduction of NGS platforms like the Genome Sequencer 20 (GS20) by 454 Life Sciences, marking the dawn of high-throughput genomic analysis (Kulski 2016). Moreover, the techniques such as Southern blotting, northern blotting, chromosome walking, and genetic transfer enabling precise isolation, characterization, and modification of genetic material, have also been employed in molecular diagnosis (Boerman et al. 2001). These molecular approaches have enhanced laboratory diagnostics, offering predictive and prognostic insights. Today, it is possible to identify individuals at risk for certain diseases before symptoms emerge, providing early indications of potential health issues. Over the past 50 years, clinical laboratories have evolved into sophisticated, technology-driven institutions focused on disease diagnosis, health monitoring, and detection of physiological abnormalities in both humans and animals.

The advent of next-generation sequencing (NGS) and bioinformatics has propelled molecular diagnostics to new heights. Common molecular diagnostic techniques now include fluorescence in situ hybridization (FISH), PCR, microarrays, MALDI-TOF, ELISA, and nucleotide sequencing. FISH allows for the detection of specific nucleic acid sequences via fluorescently labeled probes, which are commonly used in the diagnosis of genetic features in tumors, cancers, and amniotic fluid samples. PCR remains widely employed for rapid amplification of DNA or RNA, essential for pathogen detection, SNP analysis, and other diagnostic applications. Microarrays offer high-throughput screening of large genetic datasets, facilitating chromosomal abnormality detection, gene expression analysis, and mutation profiling, with companies such as Illumina and Affymetrix leading innovations in this space. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) enables genotyping and somatic mutation profiling, while enzyme-linked immunosorbent assay (ELISA) is extensively used to detect hormones, peptides, proteins, and antibodies, playing a critical role in diagnosing diseases like ovarian and breast cancer, HIV, and West Nile virus.

The COVID-19 pandemic catalyzed unprecedented advancements in molecular diagnostics. Traditional methods like computed tomographic (CT) scans and RT-PCR were foundational in early detection but had limitations in sensitivity and speed, especially during the early stages of infection (Aminian 2020; Nolan 2006). The urgent demand for rapid and cost-effective diagnostics spurred the development of innovative approaches. Techniques such as RT-LAMP, SHERLOCK, and aptamer-based diagnostics became essential for large-scale detection. Portable, point-of-care (POC) devices enabled population screening outside of hospital settings, transforming diagnostic capabilities in the pandemic context. Previous viral outbreaks, including SARS-CoV and MERS-CoV, paved the way for rapid genomic diagnostics, which played a critical role in controlling the spread of COVID-19. Nucleic acid-based methods, coupled with antibody and antigen diagnostics, advanced our understanding of the virus. Although research in peptide- and aptamer-based biosensors is still in its early stages, these technologies hold promise for

enhancing diagnostic sensitivity and specificity. The pandemic has reshaped the scientific landscape, elevating the importance of molecular diagnostics through unprecedented global collaboration (Alamri 2022). One key area within molecular diagnostics is the development of genetic assays, which are increasingly used to assess individual responses and suitability for pharmaceutical treatments. The rapid expansion of molecular diagnostics has made it a cornerstone of clinical laboratory operations (Hall et al. 2003; Debnath et al. 2010).

1.7 Roadblocks in Development and Use of Molecular Diagnostics

Clinical molecular genetics has become a cornerstone of global healthcare, with many healthcare facilities establishing dedicated molecular diagnostic departments. However, the implementation of molecular diagnostics remains limited, primarily utilized for diagnosis, carrier screening, and prenatal testing, rather than for broader population-wide screening. A significant barrier to expanding this usage is the lack of cost-effectiveness analyses (Snyder et al. 2014), which hampers the understanding of the economic viability of these tests.

A major roadblock in advancing molecular diagnostics is the challenge of selecting the appropriate technology and variant detection platform. Although various methods for variant detection exist, the effectiveness of each platform is contingent on its compatibility with the laboratory's expertise, resources, and diagnostic requirements. The multitude of detection methods can complicate the selection process, with DNA sequencing—especially next-generation sequencing (NGS)—often being deemed the gold standard for identifying both pathogenic and benign DNA variations. Critical considerations include sequencing coverage, enrichment methods, and the specific genes targeted for analysis, as well as the initial investment costs and projected test volumes. Furthermore, costs related to necessary hardware, software, and testing reagents must be factored in. The training of laboratory personnel is another crucial aspect influencing the success of molecular diagnostics. Continuous education is vital, particularly in emerging areas such as pharmacogenomics (Reydon et al. 2012; Kampourakis et al. 2014), to ensure accurate interpretation of genetic data. This is especially critical in situations like prenatal or preimplantation diagnosis, where irreversible decisions depend on the outcomes of these tests. Additionally, ongoing training and proficiency testing have been shown to significantly reduce diagnostic errors, thereby enhancing the reliability of genetic interpretations.

Legal and ethical issues pose substantial hurdles as well. Reimbursement for diagnostic costs is a prominent challenge, with insurance coverage limited to a small number of tests. The absence of a comprehensive regulatory framework complicates matters further; while appropriate regulations could increase test volumes, they could also prevent unregulated genetic testing. Informed consent is another ethical concern, particularly regarding incidental findings from NGS (Roche and

Berg 2015), emphasizing the necessity for patients to understand the implications of their genetic test results. Intellectual property (IP) issues substantially impede the commercialization of molecular diagnostics. The existence of patents on many clinically significant genes complicates laboratory operations, often leading to high royalty fees that inflate reagent costs and limit access. Additionally, exclusive sublicenses can create monopolies, further restricting options for clinical laboratories. Addressing these IP challenges may require partnerships with technology holders to develop and distribute assays while ensuring compliance with patent and regulatory standards.

The formal recognition of medical genetics as a speciality is urgently needed. Although medical genetics has only recently been established as a formal medical specialty in the United States and Europe, its full implementation continues to face considerable obstacles. These challenges could delay the integration of genetic testing into routine clinical practice (Patrinos et al. 2017). In the rapidly evolving field of molecular diagnostics, the integration of advanced technologies has significantly improved disease detection and patient care, yet challenges remain. The complexity of interpreting extensive genetic data, coupled with the need for rigorous validation protocols, poses significant obstacles for clinicians and researchers alike. These factors highlight the ongoing need for innovation in diagnostic accuracy and the establishment of standardized methodologies to ensure reliable clinical applications.

Another pressing challenge is securing reimbursement for molecular diagnostic tests. Insurance providers often require substantial evidence of a test's clinical utility before agreeing to cover its costs, which can limit patient access to these advanced diagnostics. Consequently, the financial burden frequently falls on patients, restricting the broader implementation of innovations in healthcare. While NGS has revolutionized molecular diagnostics by enabling comprehensive genomic analyses, it presents its own set of challenges. The technology requires considerable computational resources and sophisticated data analysis tools to process the vast quantities of data generated. Furthermore, skilled bioinformaticians and geneticists are essential for ensuring accurate data interpretation, which can be a significant barrier in resource-limited settings. Ethical challenges, particularly concerning genetic discrimination and privacy, also pose significant roadblocks. Questions arise about who has access to an individual's genetic information and its potential misuse by employers or insurers, leading to unfair treatment based on genetic predispositions. Establishing robust privacy policies and ethical guidelines is critical to protecting patients' rights and fostering trust in molecular diagnostic technologies. There is an urgent need for enhanced education among healthcare providers about the benefits and limitations of molecular diagnostics. Misinterpretation of test results can lead to incorrect treatment decisions, particularly when patients receive targeted therapies based on flawed genetic profiling. Ongoing training and education on molecular diagnostic techniques are vital for ensuring that clinicians accurately interpret results, communicate findings to patients, and make informed treatment decisions. Improving healthcare providers' understanding of these tools can significantly enhance patient outcomes while minimizing the risks associated with misdiagnosis.

The availability of molecular diagnostics is markedly uneven across regions, resulting in significant disparities in healthcare access. In many low-resource settings, even basic molecular tests are often unavailable, with advanced assays like NGS being particularly out of reach. This lack of access can delay diagnosis and treatment for diseases that could benefit from molecular insights, such as cancer and genetic disorders. Addressing these disparities necessitates focused efforts to enhance infrastructure, train healthcare professionals, and implement equitable access policies for molecular diagnostic technologies. By addressing these roadblocks, the field of molecular diagnostics can progress and unlock the potential of personalized medicine. Collaborative efforts from policymakers, healthcare providers, and the scientific community are essential to overcoming these challenges, paving the way for more accurate diagnoses and effective treatments. Despite the promise of personalized medicine, significant hurdles remain, including high costs associated with genetic testing, the need for robust clinical evidence to validate biomarkers, and ethical considerations surrounding genetic data privacy. Successfully navigating these barriers will be crucial for integrating personalized medicine into standard healthcare practices. As the field of molecular diagnostics continues to advance, particularly with the rise of next-generation sequencing and increased accessibility to diagnostic tools, the potential to enhance the precision of personalized medicine expands. This evolution offers hope for more effective and less toxic treatments, as the integration of molecular diagnostics into clinical practice increasingly moves toward the goal of delivering the right drug to the right patient at the right time. Such a transformation not only improves patient outcomes but also optimizes healthcare resources by customizing therapies to individual needs.

1.8 Conclusion

In conclusion, the integration of molecular diagnostics into the landscape of viral disease management represents a significant advancement in the field of healthcare. As viral infections continue to pose substantial threats to public health and socioeconomic stability worldwide, the adoption of molecular diagnostic techniques is increasingly essential for timely and accurate disease identification. Despite the advantages that molecular diagnostics offer over conventional methods, several challenges remain, including high costs, regulatory complexities, and ethical concerns surrounding patient data privacy. Addressing these roadblocks is crucial for maximizing the benefits of molecular diagnostics and ensuring equitable access to these vital technologies. Future efforts should focus on fostering collaboration among stakeholders, enhancing infrastructure, and promoting education among healthcare providers to facilitate the integration of molecular diagnostics into routine clinical practice. By overcoming these challenges, the healthcare community can harness the full potential of molecular diagnostics to improve patient outcomes and effectively respond to the evolving landscape of viral diseases.

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Chapter 2
Molecular Diagnosis of COVID-19: Challenge and Pitfalls

Mohammad Gani Afridi, Paras Jain, and Aditya Arya

Abstract Molecular diagnosis has become the gold standard for detecting infectious diseases, with its demand surging during the SARS-CoV-2 pandemic, particularly in regions with mandatory testing. These diagnostics primarily analyze nucleic acids, proteins, and biomolecular conjugates. For SARS-CoV-2, the focus was on detecting viral genes or their products, with nucleic acid testing (NAT), especially real-time polymerase chain reaction (RT-PCR), being the most widely used method. Rapid antigen and antibody tests were also employed but were secondary to reverse transcription-based techniques. The pandemic underscored the need for rapid, accurate, and scalable diagnostics to curb the spread of SARS-CoV-2. Time constraints occasionally necessitated alternative approaches like flow cell-based rapid tests or loop-mediated isothermal amplification (LAMP). Advances in genetic engineering, such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, have further revolutionized diagnostics by offering precise and efficient tools for genome targeting and disease detection. CRISPR's guide RNA technology is now emerging as a promising next-generation diagnostic tool. The lessons learned from the COVID-19 pandemic have provided valuable insights for improving molecular diagnostic technologies. This chapter will explore various diagnostic approaches, including RT-PCR, LAMP, CRISPR, and rapid tests, while addressing the challenges and future prospects of these methods.

Keywords COVID-19 · Molecular diagnosis · Nucleic acids · RT-PCR · LAMP · CRISPR · LFA

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2.1 Introduction

COVID-19 is a contagious disease spread by SARS CoV-2 (previously called coronavirus). In December 2019, China reported initial cases of SARS-CoV-2 and later declared it an outbreak. February 11, 2020, the International Committee on taxonomy of viruses (ICTV) named the virus 2019-nCoV/SARS-CoV-2 and the World Health Organization (WHO) named the disease coronavirus disease 2019 (COVID-19). On March 11, 2020 COVID-19 was declared a pandemic due to its global spread. SARS CoV-2 belongs to the beta coronavirus family (+ss RNA), which includes severe acute respiratory syndrome 2003 (SARS) and Middle East Respiratory Syndrome 2012 (MERS) viruses (Zhou et al. 2020a, b). This virus primarily affects the respiratory system, later recognized as multi organ disease with a wide range of clinical problems. The disease is typically characterized by clinical symptoms, fever, cough, dyspnea, fatigue, and pneumonia like indication in radiological tests (Huang et al. 2020). This virus has the potential for causing acute respiratory distress syndrome (ARDS), which involves a severe immune response known as cytokine storm syndrome, which leads to severe pulmonary inflammation, fluid leakage, and ultimately causing respiratory failure. Respiratory and/or cardiac failure, and sepsis due to secondary bacterial infection are major causes of mortality due to COVID-19 (Wu et al. 2020; Wang et al. 2020a, b; Huang et al. 2020; Zhou et al. 2020a, b).

If we look slightly deeper into the genetic makeup and pathology of the SARS CoV-2 infections, we would better understand the underlying principles of its molecular diagnosis. The genome length of SARS CoV-2 ranges from 26 to 32 kb, has a variable number of Open Reading Frame (ORFs), and the genomic organization of the virus is similar to the known other HCoVs (Song et al. 2019). SARS CoV-2 is an enveloped virus with numerous spikes, and it possesses an unsegmented single-stranded positive-sense RNA genome. It has a 5′ cap and 3′ poly (A) tail, which allows it to act as functional mRNA for synthesis of replicase polyprotein (Wang et al. 2020a, b). The two-thirds of the viral genomic region, i.e., 20 kb lying downstream to 5′ end, possesses replicase gene referred to as Open Reading Frame 1ab (ORF1ab), which encodes the non-structural proteins (NSPs) referred to as polyprotein (pp1a) and (pp1ab), respectively. The ORF1a non-structural protein corresponds to NSP1 to NSP11 and the ORF1ab non-structural protein comprises NSP12 to NSP16. The remaining 10 kb region, located upstream of the 3′ end, encodes various structural proteins containing spike, envelope, membrane, and nucleocapsid protein. Furthermore, the structural genes encode nine accessory proteins, encoded by ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14, and ORF10 genes (Nelson et al. 2020). Additionally, the genomic region immediate to 5′ end contains two different domains, leader sequence and untranslated region (UTR); it is capable of forming a large number of stem loop structures essential for replication and transcription of SARS CoV-2 genome. The transcriptional regulatory sequences (TRSs) introduce each structural and accessory gene which are essential for their functional expression. An infectious SARS CoV-2 virion (~100 nm

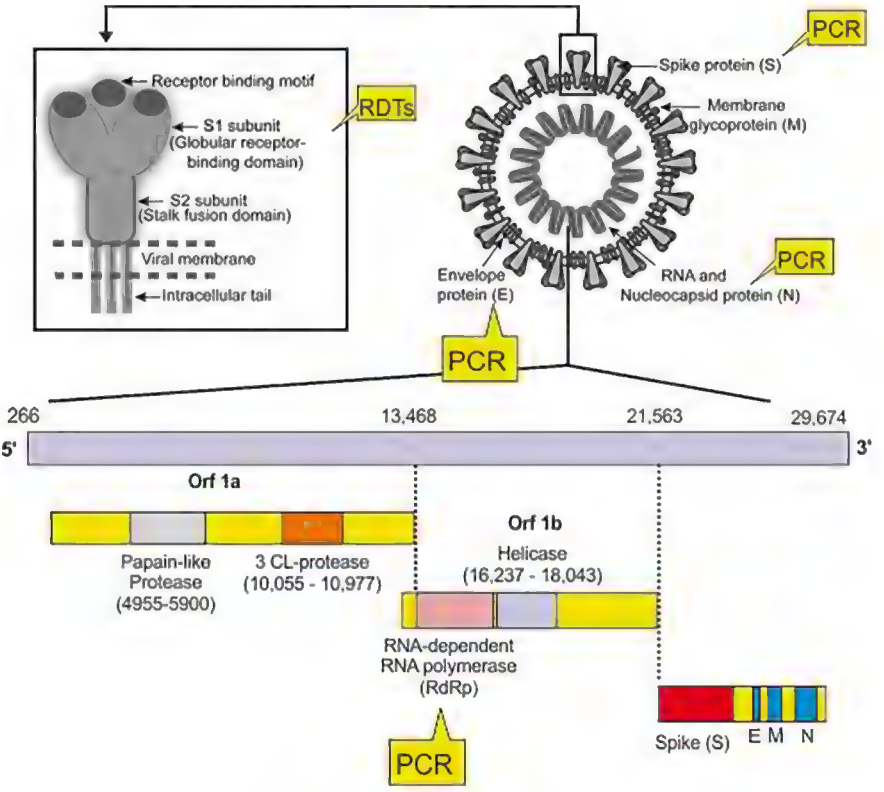

Fig. 2.1 SARS-CoV-2 genomic and proteomic makeup and potential targets for diagnosis

in diameter) contains 29903 nucleotides. Whole genome of SARS CoV-2 contains four structural and 25 non-structural proteins: envelope (E), nucleocapsid (N), membrane (M), and spike (S) proteins (Letko et al. 2020; Hoffmann et al. 2020) (Fig. 2.1). The first COVID-19 diagnosis kit protocol was published on January 23, 2020. The targeted assay genes related to nucleocapsid (N), envelop (E), and RNA-dependent RNA polymerase (RdRp) protein. RdRp was found to be the most sensitive among the various strains. These assays contain 2 probes, one probe called "Pan Sarbeco probe" detects bat-related SARS coronavirus and another coronavirus. However, another probe called RdRp-p2 was specific to SARS CoV-2 only (Chan et al. 2020). The genome of the virus is encapsulated with a lipid envelope studded with spike (S) protein. This S protein plays a key role in viral entry into the host cell which contains the ACE-2 receptor (Letko et al. 2020; Hoffmann et al. 2020). These ACE-2 receptors are prominently found on respiratory epithelial cells and other tissues. This binding facilitates the entry and replication of virus, leading to cytopathic effect and a subsequent inflammatory response (Carsana et al. 2020). COVID-19 can trigger cytokine storms, where elevated levels of pro-inflammatory cytokines like tumor necrosis factor (TNF-α), interleukin 6 (IL-6) and (IL-1β) are observed.

This systemic inflammation can lead to tissue damage, multi-organ failure, and increased mortality (Mehta et al. 2020). Not only pulmonary, COVID-19 affects the various other organs. The cardiovascular system is frequently involved in myocarditis and pericarditis to thromboembolic events. Renal involvement, evidenced by acute kidney injury and linked to poor prognosis. It also affects the central nervous system and causes strokes, encephalitis, and neurological complications (Gupta et al. 2020). To combat SARS CoV-2 infection, the host immune system plays a pivotal role in this. Innate immunity, such as interferon signaling and inflammatory cytokine production, activates early to limit the spread of viral infection. However, dysregulated immune responses may also contribute tissue damage and disease severity. In addition, adaptive immunity produces the neutralizing antibodies and the activation of T cells; it plays a major role in clearing the virus and developing long-term immunity (Zhu et al. 2020; Tay et al. 2020; Walls et al. 2020).

After infection with SARS CoV-2, innate immunity cells recognize viral particles through pattern recognition receptors (PRRs). PPRs are expressed in immune cells, including monocytes, neutrophils, macrophages, dendritic cells, natural killer (NK) cells, mast cells, eosinophils, basophils (Gasteiger et al. 2017), and start rapid inflammatory response, characterized by the production of chemokines and cytokines (IL-6 and TNF-α) that recruit immune cells to the site of infection. Dysregulated cytokine response, also known as cytokine storm, can contribute to severe disease manifestations and tissue damage. Furthermore, adaptive immune response, including the production of neutralizing antibodies that targets the spike protein and activation of T cells specific to the viral antigen, play a crucial role in SARS CoV-2 clearance and recovery.

Whole genome sequencing (WGS) of SARS CoV-2 led scientists to develop testing protocols for diagnosis of pathogens and also provided an insight in the phylogenetic study of viruses. During the initial period of epidemic, complete sequencing of SARS CoV-2 facilitated the specific laboratory diagnostic kit by designing specific primers (Lu et al. 2020a). Insights from genomic investigations, clinical observations, and epidemiological data are crucial for improving public health initiatives and therapeutic interventions as researchers work to understand the complexity of COVID-19 pathophysiology and molecular biology. The quick rollout of vaccinations and the development of sensitive diagnostic tests like real-time polymerase chain reaction (RT-PCR), LAMP assay, and flow cell-based techniques highlight how important molecular knowledge is in the fight against the pandemic.

2.2 Tools and Approaches for Molecular Diagnosis

Generally speaking, molecular diagnostics encompasses the majority of diagnostic techniques and instruments designed to identify a distinctive biomarker—specifically, nucleic acids—in a sensitive and targeted way. Since nucleic acid detection is the primary focus of these techniques, a significant portion of molecular diagnostic tools for SARS-CoV-2 concentrate on amplifying and detecting virus-specific

genetic material or its components (RNA). Nucleic acid amplification techniques (NAAT) is a common term used to describe this collection of methods. Real-time RT-PCR is the most appealing option for NAAT and was the gold standard for molecular diagnosis of SARS-CoV-2 throughout the pandemic. The loop-mediated isothermal amplification (LAMP) assay is a reasonably straightforward but sensitive technique that is also recommended for a quick infection detection. In addition to NAAT, various protein-based techniques served as the foundation for comparatively quicker point-of-care (POC) detection (Table 2.1). The majority of POCs, or fast diagnostic methods, rely on the utilization of immobilized antibodies or antigens and lateral flow. Figure 2.2 summarizes various well-established and emerging approaches in molecular diagnosis of COVID-19.

2.3 Conventional Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

In the initial days of COVID-19 pandemic, several conventional approaches of PCR were followed primarily due to limited supplies and non-availability of probes. In the conventional diagnostic PCR, the RNA extraction of nasopharyngeal swab extract followed by amplification of gene targets of the virus. Although the reverse transcription and amplification is well-established approach in research and occurs with a high specificity, yet it requires multiple steps, trained personnel, and specialized equipment, making it less suitable for rapid and high-throughput diagnostic settings.

2.4 Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR (or qRT-PCR) is the one of the most widely used method to detect SARS CoV-2 and also known as the gold standard technique for detecting SARS-CoV-2. Virus can be detected by amplifying its genetic material from patient samples (Tang et al. 2020). Real time RT-PCR involves converting SARS CoV-2 RNA into c-DNA using an enzyme known as reverse transcriptase. After the c-DNA synthesis it starts the amplification using polymerase chain reaction with the help of a specific designed primer and probe to target SARS CoV-2 genes. Fluorescent dye attached to the probes helps in real time detection via emit signals in form of light, each cycle of amplification measured on the basis of wavelength, allowing for both qualitative and quantitative viral RNA analysis (Corman et al. 2020). The COVID-19 RT-PCR diagnosis typically targets several specific genes from the SARS CoV-2 genome to ensure accuracy and reliability. The targeted assay genes related to nucleocapsid, envelope, RNA-dependent RNA polymerase (RdRp) protein. RdRp

Table 2.1 Summary of widely tested and emerging diagnostic methods for COVID-19, including their target genes, pros, and cons

Methods		Target genes	Pros	Cons
Nucleic acid amplification assays (NAAT)	Reverse transcriptase polymerase chain reaction (RT-PCR)	ORF1ab, N, E, S genes	High sensitivity and specificityGold standard for diagnosisDetects active infections	Requires specialized equipmentExpensiveLonger turnaround time (several hours)Cold chain required for sample transport
	Digital PCR (dPCR)	ORF1ab, N, E genes	High sensitivityQuantitative resultsUseful for low viral load samples	ExpensiveRequires specialized equipment and expertise
	Loop-mediated isothermal amplification (LAMP)	ORF1ab, N, E genes	Faster than RT-PCRNo thermal cycling requiredCan be performed in decentralized settings	Moderate sensitivityRequires trained personnelLimited adoption compared to RT-PCR
	Cartridge-based nucleic acid amplification (CBNAAT)	ORF1ab, N, E genes	Faster than RT-PCRCan be performed in decentralized settings	Moderate sensitivityRequires trained personnelLimited adoption compared to RT-PCR
Emerging molecular approaches	Next-generation sequencing (NGS)	Entire SARS-CoV-2 genome	Provides comprehensive genetic informationUseful for identifying variants and mutations	Expensive and time-consumingRequires specialized infrastructure and expertise
	CRISPR-based tests (e.g., SHERLOCK, DETECTR)	ORF1ab, N genes	Highly specificRapid resultsEasy to adapt for different targets	Requires specialized reagents and equipmentStill under development for widespread use
Immunological/lateral flow assays	Serological tests (antibody detection)	IgM, IgG against viral antigens	Identifies past infectionsUseful for seroprevalence studiesCheaper and simpler than molecular tests	Cannot detect active infectionsDelay in antibody production (7–14 days post-infection)Not useful for early-stage detection
	Rapid antigen test (RAT)	Viral nucleocapsid protein	Quick results (15–30 minutes) Cost-effectiveEasy to use in point-of-care settings	Lower sensitivity compared to RT-PCRHigh chance of false negativesLess effective in asymptomatic or early infections
Imaging[a]	Chest CT imaging[a]	Not gene-specific	Useful for identifying lung abnormalities in severe casesCan complement molecular tests	Non-specific (cannot confirm COVID-19)High costNot suitable for mass testing

[a]Not considered as molecular diagnostic test, only used here for reference

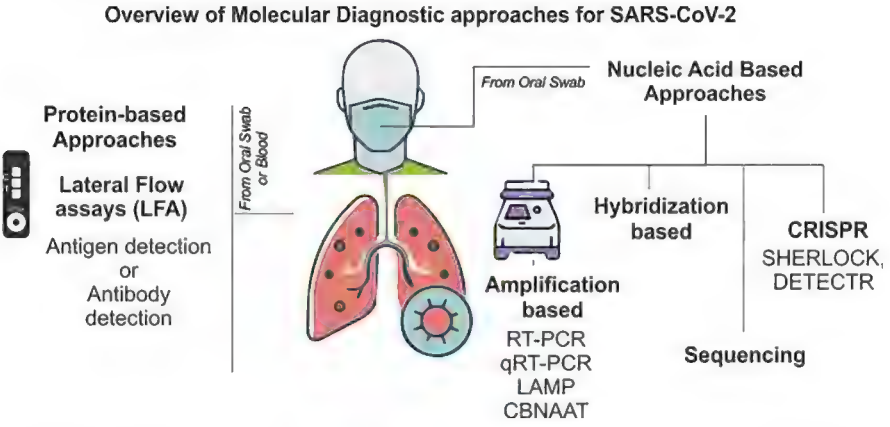

Fig. 2.2 Various conventional and emerging approaches for molecular diagnosis of SARS-CoV-2

was found to be the most sensitive among the various strains. These assays contain 2 probes, one probe called Pan Sarbeco probe detects bat-related SARS coronavirus and another coronavirus. However, another probe called RdRp-p2 was specific to SARS CoV-2 only (Chan et al. 2020). Commonly targeted genes include

1. *N Gene:* This gene encodes the nucleocapsid protein and is highly conserved among SARS coronaviruses and other coronaviruses. It is a primary target due to its high abundance in the virus. This gene is also known as the screening gene.
2. *E Gene:* The envelope protein codes by this E gene and is used as a screening target in some RT-PCR assays.
3. *S Gene:* This screening gene is crucial for viral entry in the host and codes the spike protein. Some assays include this gene as a target to increase the sensitivity of detection.
4. *RdRp Gene:* RNA-dependent RNA polymerase enzyme is encoded by this RdRp gene, an essential enzyme for viral replication. This gene is highly specific to SARS-CoV-2 and is often used in confirmatory tests.

Diagnosis of COVID-19 using RT-PCR starts with the respiratory sample collection. Nasopharyngeal or oropharyngeal samples taken through swab stick in viral transport medium (VTM). VTM contains antibiotic, antifungal for inhibiting bacterial and fungal contaminants that could compromise the integrity of virus sample and hydroxyethyl piperazine ethane sulfonic acid (HEPES) protects virus particles to degrade and it maintains pH during transport and storage of viral samples (Deng et al. 2020; CDC 2020). The collected sample RNA extracted using commercial and automated systems (Tang et al. 2020). Extracted RNA is then added into RT-PCR kit mater mix. As SARS CoV-2 is RNA virus, it is first reverse transcribed c-DNA using reverse transcription then c-DNA amplified using specific primer probes in the thermal cycler. The amplification is monitored in real time via fluorescence signals. The presence of viral nucleic acid is determined on the fluorescence signals emitted during the amplification cycles. A positive signal indicates the presence of

SARS-CoV-2 RNA. Ct value or cycle threshold value, represents the cycle number at which the fluorescent signal of PCR product crosses a defined threshold, at which the target viral gene is detectable. It is generally conceived that the Ct value is inversely proportional to the targeted genetic material in the sample; the lower Ct means the higher amount of viral RNA present (Corman et al. 2020), provided that the sample collection was performed uniformly, though it is not straightforward and directly conclusive of viral load or copy number.

2.4.1 Sensitivity and Specificity of PCR-Based Diagnosis

The sensitivity of RT-PCR refers to the ability of RT-PCR to correctly diagnose individuals who are infected with that particular pathogen or not. The RT-PCR test sensitivity varies in SARS-CoV-2 detection throughout the infection time period. Starting at 0% about 8 days pre-symptom onset, sensitivity significantly rises to 86% on the symptom day and peaks at 95% approximately 5 days post onset, remaining consistently high (>87%) from 5 to 14 days after infection. It declines faster in vaccinated individual, female, younger individual, overseas-acquired cases, and pacific populations. These findings emphasize the test's reliability in diagnosing COVID-19 across different demographics, crucial for clinical management and public health strategies (Rachelle et al. 2022).

The sensitivity of RT-PCR using nasopharyngeal specimen for SARS CoV-2 diagnosis, reported a pooled sensitivity of 89% (95% CI, 85.4 to 91.8%) evaluated by systematic review and meta-analysis**.** Although the emergence of alternative specimens such as saliva and nasopharyngeal swabs remain important due to their higher sensitivity for COVID-19 diagnosis (Zhen et al. 2020; Butler-Laport et al. 2021). Proper sample collection is vital for optimal test accuracy. False positive and false negative both results can impact on patient care. False positive occurs when RT-PCR assay incorrectly identifies a person as infected with COVID-19 when they are not. This can be due to cross-reactivity with other nucleic acid, contamination, or technical errors in the laboratory. While false negative happens when the RT-PCR incorrectly indicates a person is not infected with COVID-19 when they actually are. It can occur if the sample is collected too early or late in the course of infection. The viral load governs the RNA amount in the samples. The higher viral load, the more RNA will have a high chance to get true positive results. Secondly, the improper manipulation and storage might be subject to denaturation and degradation of viral RNA, the result of which lowers the final amount of intact RNA for the test (Karthik et al. 2020).

There are several significant advantages for COVID-19 diagnosis using RT-PCR assay. Firstly, RT-PCR is highly sensitive and specific, allowing for the accurate detection of SARS CoV-2 RNA even at low viral load, which is a key role for early and effective diagnosis for management of disease. RT-PCR assay can reliably detect the virus with high sensitivity and specificity, even in asymptomatic individuals or those with low viral load (Carter et al. 2020). RT-PCR can identify the

SARS-CoV-2 before symptoms appear, which results in timely isolation of patients and reducing the viral transmission. Additionally, this assay provides quantitative data on viral load, by which we can monitor the progression of infection and assess the effective treatment (Roman Wolfel et al. 2020). As a review discussed in (WHO 2020) method's widespread available and established protocol make it a cornerstone in diagnostic laboratories worldwide. It offers rapid results, often within a few hours, which is crucial for timely patient and public health management (Kucirka et al. 2020). These qualities make RT-PCR a robust and essential tool in battle against COVID-19.

The high sensitivity and specificity of RT-PCR make it robust; it has several limitations for COVID-19 diagnosis. One of the biggest challenges is the potential for false-positive results, particularly issue during sample collection and when the viral load is low or and the sample collection early or late in the infection. This can lead to missed diagnoses or false negatives and hinder efforts to control virus transmission (Kucirka et al. 2020). Additionally, this needs sophisticated laboratory equipment and trained personnel, making it less accessible in resource limited settings or during outbreaks with high testing demand. Although the turnaround time of the results is generally rapid within a few hours, and it may vary depending upon the laboratory capacity and testing volume, which may delay timely patient and public health interventions (Wiersinga et al. 2020). Additionally, it does not indicate the presence of infectious virus, as RT-PCR detects genetic material rather than live virus, posing challenges for identifying infective and guiding isolation protocols (Wolfel et al. 2020). Despite these limitations, RT-PCR remains a cornerstone in COVID-19 diagnostics, requiring careful interpretation alongside clinical and epidemiological data to maximize its utility in controlling the pandemic. A variety of kits for the PCR-based diagnosis are available, some of which have been enlisted in Table 2.2 with respective description.

2.5 Digital Droplet PCR

Digital Droplet PCR (ddPCR) is a highly sensitive and precise molecular technique increasingly used in the diagnosis of COVID-19. Unlike traditional RT-PCR, ddPCR partitions the nucleic acid sample into thousands of nanoliter-sized droplets, each serving as an individual PCR reaction. This droplet-based format allows for absolute quantification of SARS-CoV-2 RNA without the need for standard curves, making it particularly effective in detecting low viral loads. ddPCR targets specific viral genes, such as N, E and ORF1ab, ensuring robust detection even in challenging samples like those with degraded RNA or during late-stage infections with minimal viral shedding. Prominent ddPCR kits include the *Bio-Rad SARS-CoV-2 Droplet Digital PCR Kit*, which is validated for high precision, and the *RainSure COVID-19 ddPCR Detection Kit* by RainDance Technologies, known for its multiplexing ability. The primary advantages of ddPCR include its exceptional sensitivity, enabling early diagnosis and monitoring of viral load dynamics, which is critical

Table 2.2 Summary of a few widely used real time RT-PCR kits for molecular diagnosis of COVID-19 (Not all kits have been included and the table neither it makes a comparison of kits, nor provides any preference for use)

Manufacturer	Kit name	Target genes	Turnaround time	Sample type	Detection limit	Special features
Thermo Fisher Scientific (USA)	TaqPath COVID-19 Combo Kit	ORF1ab, N, S	~1–2 hours	Nasopharyngeal, oropharyngeal	10 copies/ reaction	Multiplex assay; high specificity; validated globally
Roche Diagnostics (Switzerland)	cobas® SARS-CoV-2 Test	ORF1, E	~3 hours	Nasopharyngeal, oropharyngeal	<100 copies/ mL	High throughput on cobas® 6800/8800 systems; CE-IVD marked
Abbott Molecular (USA)	m2000 RealTime SARS-CoV-2 Assay	RdRp, N	~2 hours	Nasopharyngeal, oropharyngeal	100 viral RNA copies/ mL	High precision; compatible with Abbott m2000 platform
Bio-Rad Laboratories (USA)	SARS-CoV-2 RT-PCR Detection Kit	ORF1ab, N	~90 minutes	Nasopharyngeal, oropharyngeal	Not specified	Reliable with Bio-Rad CFX96 system; user-friendly software
Qiagen (Germany)	QIAstat-Dx Respiratory Panel 2	Multiple respiratory genes	~1 hour	Nasopharyngeal, oropharyngeal	<1 copy/μL RNA	Multiplex panel for SARS-CoV-2 and other respiratory pathogens; fast and compact
Molbio Diagnostics (India)	Truenat Beta CoV & SARS-CoV-2	E, RdRp	~1 hour	Nasopharyngeal, oropharyngeal	~100 copies/ reaction	Portable and battery-operated; field-friendly; ideal for low-resource settings
PerkinElmer (USA)	SARS-CoV-2 Real-time RT-PCR Kit	ORF1ab, N	~90 minutes	Nasopharyngeal, oropharyngeal	<10 copies/ reaction	High sensitivity and specificity; validated by multiple regulatory agencies
Primerdesign (UK)	Genesig® COVID-19	ORF1ab	~90 minutes	Nasopharyngeal, oropharyngeal	20 copies/ reaction	CE-IVD marked; customizable options for labs with different throughput requirements
Sansure Biotech (China)	Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit	ORF1ab, N	~90 minutes	Nasopharyngeal, oropharyngeal	500 copies/ mL	Widely used in China and other countries; compatible with standard RT-PCR platforms
Seegene (South Korea)	Allplex™ SARS-CoV-2 Assay	E, N, RdRp	~2 hours	Nasopharyngeal, oropharyngeal	250 copies/ mL	High multiplexing capability; scalable for high-throughput testing

for managing asymptomatic cases or assessing treatment efficacy. Moreover, ddPCR exhibits reduced susceptibility to PCR inhibitors, making it suitable for diverse sample types, including saliva and environmental specimens. However, ddPCR has limitations, such as higher costs, longer turnaround times compared to conventional RT-PCR, and the need for specialized equipment and technical expertise, restricting its widespread use in resource-limited settings. Despite these challenges, ddPCR represents a valuable tool in COVID-19 diagnostics, particularly for applications requiring high sensitivity and accuracy, such as evaluating vaccine efficacy, tracking low-level mutations, or conducting epidemiological studies in low-prevalence populations.

2.6 Reverse Transcription Loop-Mediated Isothermal Amplification Assay (RT-LAMP)

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a molecular diagnostic tool becoming more attractive because it does not require a thermocycler (Yan et al. 2020). It got significant attention for detection of SARS CoV-2 responsible for COVID-19. Hence RT-LAMP is cheaper than RT-PCR with comparable sensitivity (Lu et al. 2020a). RT-LAMP is developed by the combination of two techniques: reverse transcription and LAMP. It enables the RNA sequence amplification at a constant temperature, which makes the process easier compared to traditional PCR methods (Notomi et al. 2000). RT-LAMP assay results can be visualized either via colorimetric or fluorometric detection methods providing clear indications of the presence or absence of the target sequence (Goto et al. 2009; Mori and Notomi 2009).

The RT-LAMP process is started with reverse transcription of viral RNA into c-DNA using reverse transcriptase enzyme, the c-DNA undergoes isothermal amplification that requires constant temperature (60–65 °C). This assay employs a set of four to six primers [Forward Outer Primer (F3), Backward Outer Primer (B3), Forward Inner Primer (FIP), Backward Inner Primer (BIP), Loop Forward Primer (LF), Loop Backward Primer (LB)], ensures the high specificity that recognizes six distinct sequences of the targeted DNA. During the amplification, a *Bst* 2.0 DNA polymerase enzyme (New England Biolabs) is used in the reaction with strand displacement activity and reverse transcriptase activity and synthesizes new c-DNA strands, leading to the exponential amplification of targeted DNA. There are various methods for detection of amplified products, such as colorimetric changes, turbidity or fluorescence, which allows the rapid and easy interpretation of the results. The high specificity makes RT-LAMP a powerful tool for rapid and accurate diagnosis of COVID-19 (Mori and Notomi 2009).

2.7 The Cartridge-Based Nucleic Acid Amplification Test (CBNAAT)

The Cartridge-Based Nucleic Acid Amplification Test (CBNAAT) is a rapid, highly sensitive, and automated molecular diagnostic technique used for the detection of SARS-CoV-2, the causative agent of COVID-19. This test employs real-time polymerase chain reaction (RT-PCR) integrated within a compact cartridge system, which minimises the need for manual handling and reduces the risk of contamination. CBNAAT targets specific regions of the viral RNA, such as the N and E genes, using primers and probes preloaded into the cartridge. Upon introduction of a patient sample, typically nasopharyngeal or oropharyngeal swabs, the device performs RNA extraction, amplification, and detection in a single, self-contained system. Results are typically available within 45 minutes to an hour, making CBNAAT particularly valuable in point-of-care and emergency settings where rapid diagnosis is critical.

A key advantage of CBNAAT is its simplicity and portability, which allows deployment in decentralized healthcare facilities, including rural and low-resource settings. The method's high specificity and sensitivity align closely with traditional RT-PCR standards, making it reliable for confirming COVID-19 infections. Additionally, the automation reduces the need for skilled laboratory personnel and complex laboratory infrastructure. However, CBNAAT systems, such as GeneXpert, require specialized cartridges that are costly and subject to supply chain constraints, limiting their scalability in large-scale testing scenarios. Despite these limitations, the technology has proven instrumental in augmenting diagnostic capacity during the pandemic, particularly for rapid case identification and containment efforts.

2.8 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas System

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology is a revolutionary technology that was originally discovered in prokaryotes that allows for precise editing of DNA. These sequences were first identified in *Escherichia coli* K-12, by Dr. Yoshizumi Ishino and his team in 1987, in the course of the analysis of the gene responsible for isozyme conversion of alkaline phosphatase (Ishino et al. 1987). And since then, these have been discovered in different prokaryotes (Mojica et al. 2000), when virus infects bacteria, the repeated sequences are transcribed into CRISPR RNAs (crRNAs) which act as guide to CRISPR associated (Cas) protein to break or degrade the viral genome. Therefore, the Cas system is known as the adaptive immune system of bacteria (Jinek et al. 2012). The most common Cas9, Cas12, and Cas13 to target and modify specific sequences of the genome. The Cas protein acts as effector to recognize and degrade specific genomic targets, complimentary to the specific guide RNA (primarily a tool for genome

editing), is emerging for the development of new generation potential diagnostic, prophylactic, and therapeutic tools. Due to its specificity, design, feasibility, etc. The research has explored the potential of different CRISPR/Cas systems as a tool for development of novel diagnostics and therapeutics (Cox et al. 2017). For COVID testing, there are two most common CRISPR tools DETECTR and SHERLOCK. The researchers repurposed two enzymes, Cas12 and Cas13, as virus hunters. These enzymes get programmed to recognize bits of SARS-CoV-2 genetic material. Mechanistically, once CSIRPR-Cas lock onto the virus, it activates the degradation, thereby cleaving any nearby gene fragment. Detection may later follow, using fluorescent approaches.

2.9 Next-Generation Sequencing

Next-generation sequencing (NGS) has emerged as a powerful tool in the diagnosis and management of COVID-19, particularly in identifying and characterizing SARS-CoV-2. NGS is primarily used when a detailed understanding of the virus is required, such as in whole-genome sequencing for variant tracking, outbreak investigations or identifying novel mutations that may influence transmissibility, pathogenicity, or vaccine efficacy. Key platforms for NGS in COVID-19 include *Illumina (e.g., MiSeq, NextSeq, NovaSeq), Oxford Nanopore Technologies (e.g., MinION, GridION)*, and *Thermo Fisher Scientific Ion Torrent* systems. Illumina platforms are known for high-throughput and accuracy, making them ideal for sequencing large volumes of viral genomes. Oxford Nanopore's portable systems are better suited for field studies due to their rapid turnaround and real-time data analysis capabilities. The use of NGS in diagnostics involves amplifying and sequencing viral RNA from clinical samples to confirm SARS-CoV-2 presence and reconstruct the virus's genome. Compared to RT-PCR, NGS provides comprehensive data beyond simple detection, including insights into co-infections and host–pathogen interactions. Its advantages include unparalleled accuracy in mutation detection, scalability for large-scale genomic surveillance, and adaptability for emerging pathogens. However, NGS is limited by its high cost, complex bioinformatics requirements, and longer sample processing times compared to RT-PCR. Additionally, it requires sophisticated infrastructure and technical expertise, making it less accessible in low-resource settings. Despite these challenges, NGS plays a critical role in the pandemic response, particularly for global surveillance of variants, vaccine efficacy monitoring, and research into SARS-CoV-2 evolution.

NGS-based molecular diagnosis of COVID-19 has significantly advanced understanding of SARS-CoV-2. It enabled rapid genome sequencing in early 2020, facilitating the development of diagnostic tests and vaccines. NGS has proven critical for detecting mutations in variants of concern, such as Delta and Omicron, aiding in assessing their transmissibility, immune evasion, and clinical severity. Studies have demonstrated its ability to detect SARS-CoV-2 in low viral load cases and complex samples, enhancing surveillance and early outbreak detection. NGS also uncovers

co-infections and secondary infections in patients with COVID-19, providing insights into disease progression and treatment strategies. Its use in genomic epidemiology has traced transmission chains and monitored viral evolution. However, challenges include variability in sequencing accuracy, high costs, and limited accessibility in low-resource settings, though portable platforms like Oxford Nanopore offer promising solutions. NGS remains a cornerstone in understanding SARS-CoV-2 dynamics and supporting pandemic management efforts.

2.10 Lateral Flow Immunoassays for COVID Diagnosis

Lateral flow immunoassays (LFIs) are self-contained and function independently, thanks to their design and the use of capillary action. The device consists of multiple components made from different materials—sample pad, conjugate pad, detection pad, and absorbent pad—arranged in an overlapping configuration. This allows for continuous movement of an aqueous sample from one end of the strip to the other. When a liquid biological sample is applied to the sample pad, it travels via capillary action to the conjugate pad, which holds a detector agent. If the target analyte is present, it binds to the detector agent, which is linked to a signal generator. These immunocomplexes then reach the test line(s) on the detection pad, where capture agents are fixed. The accumulation of signal generators at the test line allows detection of the immunocomplexes, while unbound substances move further to the absorbent pad.

2.11 Future Perspectives: Opportunities and Challenges

The COVID-19 pandemic has highlighted the indispensable role of molecular diagnostics in infectious disease management, revealing both the strengths and limitations of current technologies. The future of molecular diagnostics, particularly in the context of pandemic preparedness, presents significant opportunities for advancement. Multiplex assays capable of detecting multiple pathogens simultaneously, coupled with point-of-care testing (POCT) innovations, could revolutionize outbreak management by enabling rapid, decentralized testing. Artificial intelligence (AI) integration with diagnostic platforms offers the potential to enhance both the accuracy and speed of diagnostic processes, allowing for real-time data analysis and outbreak prediction. Furthermore, the shift towards personalized medicine, where diagnostic tools can tailor treatments based on individual genetic profiles and pathogen characteristics, underscores the potential for improved therapeutic outcomes and more efficient disease control. However, realizing these advancements requires addressing several critical challenges.

Supply chain vulnerabilities exposed during the pandemic underscore the need for a more resilient and diversified supply chain for diagnostic reagents, equipment,

and consumables. Contamination risk remains a significant concern in nucleic acid-based diagnostics, necessitating the implementation of stringent quality control protocols to mitigate false-positive results. Logistical complexities associated with the rapid distribution of tests, particularly in resource-limited settings, highlight the importance of establishing efficient distribution networks. Scalability is another crucial issue; increasing testing capacity rapidly during a pandemic demands investments in flexible manufacturing processes and laboratory infrastructure. Cold chain requirements for nucleic acid-based tests pose additional logistical challenges, particularly in low-resource settings where maintaining strict temperature controls can be difficult. The risk of false negatives, especially in cases of low viral loads, necessitates the development of more sensitive assays. Rapid turnaround times are essential for effective disease control, driving the need for faster amplification techniques and automated workflows. Ensuring high standards of quality control across laboratories globally, streamlining regulatory approval processes without compromising safety, and implementing robust data management systems to handle the vast amounts of diagnostic data are all critical components of a successful future pandemic response. Addressing these challenges through innovation, investment, and international collaboration will be key to enhancing the reliability, scalability, and accessibility of molecular diagnostics in the face of future pandemics.

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Chapter 3
Choice of Samples for Molecular Diagnosis of Viral Disease

Sneha Singh, Ankit Yadav, and Swati Sharma

Abstract The selection of appropriate biological samples is crucial for the molecular diagnosis of viral diseases, as sample type significantly influences the accuracy, sensitivity, and reliability of diagnostic outcomes. This chapter provides an overview of various clinical specimens, including blood, saliva, urine, nasopharyngeal swabs, and cerebrospinal fluid, highlighting their advantages and limitations in molecular testing. Blood is widely used due to its high viral load in systemic infections, but it requires invasive collection. Saliva and urine offer non-invasive alternatives and are suitable for detecting certain viruses, though they may have lower viral concentrations. Nasopharyngeal and oropharyngeal swabs are standard for respiratory viruses like SARS-CoV-2 and influenza but may present challenges in sample collection and handling. Cerebrospinal fluid is critical for diagnosing neurotropic viral infections but necessitates specialized procedures. The chapter also discusses the role of specimen stability, transport conditions, and pre-analytical variables in optimizing molecular diagnostic efficiency. Understanding the pros and cons of different sample types allows for informed decisions in clinical and research settings, ensuring effective viral detection and disease management. The chapter concludes by emphasizing the need for standardized guidelines and emerging techniques to enhance sample collection and molecular diagnostic accuracy.

Keywords Sample type · Saliva · Nasopharyngeal swab · Urine · Blood · Molecular diagnosis

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3.1 Introduction

Over the past few years, molecular diagnostic methods for viral testing have advanced rapidly, leading to their widespread use in laboratories to detect viral pathogens in humans (Cobo 2012). Despite the advancement, accuracy of diagnostic results largely depends on the specimen used. Each sample type has its own complexities, offering both opportunities and challenges (Pretorius and Venter 2017). The type of sample required varies based on the nature of the test being performed and the virus under investigation (Louten 2016). Depending on their invasiveness of collection, samples can be broadly categorized, which in turn affects accessibility, patient compliance, and ultimately, the diagnostic accuracy.

The choice of biological samples for molecular diagnosis of viral diseases is a critical determinant of diagnostic accuracy, as it directly affects the sensitivity and specificity of detection methods. The ideal sample should maximize viral yield while minimizing patient discomfort and procedural risks. *Invasive samples*, such as blood, cerebrospinal fluid (CSF), and tissue biopsies, often provide high viral loads and are essential for detecting systemic and neurotropic infections. For instance, blood samples are routinely used for diagnosing HIV, hepatitis viruses, and cytomegalovirus (CMV) due to their ability to carry a high concentration of viral particles. Similarly, CSF is indispensable for diagnosing viral meningitis and encephalitis caused by herpes simplex virus (HSV) and enteroviruses, though its collection via lumbar puncture is invasive and requires specialized handling. However, *invasive sample* collection can cause significant pain, pose risks of secondary infections, and require skilled professionals. This limitation necessitates the exploration of non-invasive alternatives that offer comparable diagnostic accuracy. While saliva, urine, tears, and nasal secretions are easier to obtain, their effectiveness depends on the viral shedding pattern and the concentration of viral genetic material present. For example, saliva is increasingly used for detecting SARS-CoV-2 and Epstein–Barr virus (EBV), but its reliability varies across different viral infections due to potential dilution and degradation of viral RNA. The classification of samples based on invasiveness provides a systematic approach to selecting the most suitable specimen for molecular testing. *Non-invasive samples* such as urine and saliva are particularly useful in large-scale screenings and epidemiological studies, reducing the logistical burden associated with sample collection. Urine has proven effective in detecting viruses like BK virus and Zika virus, while self-collected saliva samples facilitate easier population-wide COVID-19 screening. However, non-invasive specimens may have lower viral loads and require more sensitive detection techniques such as polymerase chain reaction (PCR) with enhanced amplification cycles. On the other hand, semi-invasive samples, including nasopharyngeal swabs and bronchoalveolar lavage, serve as critical diagnostic tools for respiratory viruses but may cause patient discomfort and require trained personnel for collection (Fig. 3.1). The choice of sample must balance the need for high sensitivity with patient safety and feasibility. For molecular diagnostics to be most effective, clinicians must consider factors such as viral pathophysiology, sample stability, ease of transport, and pre-analytical processing. While the preference is to

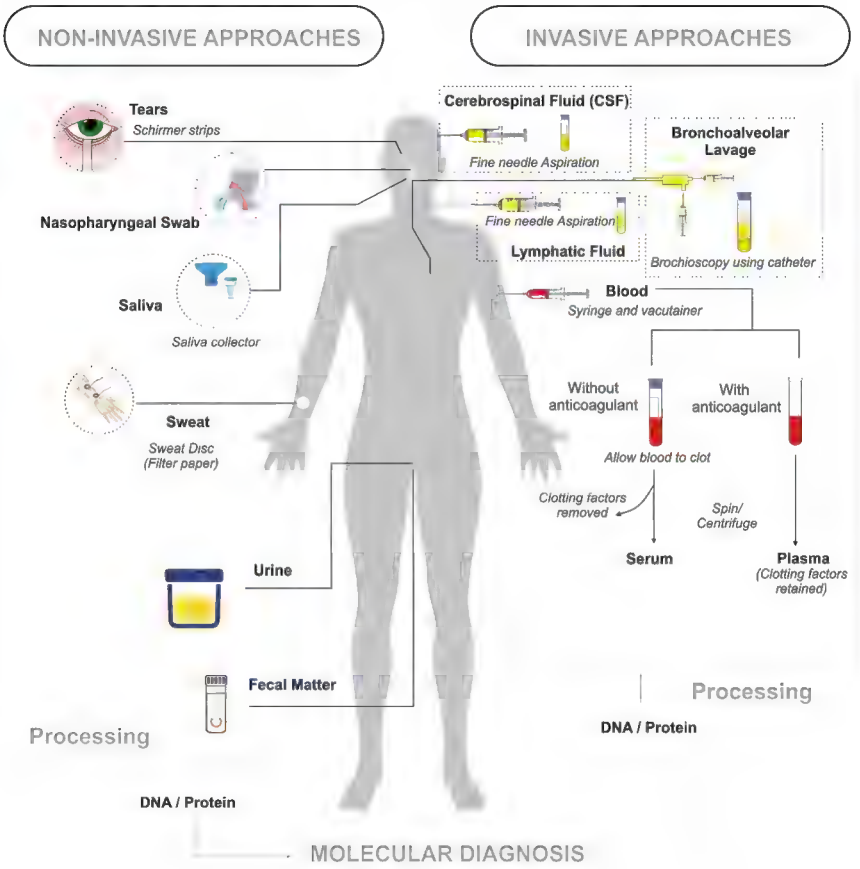

Fig. 3.1 Summary of various commonly used biological samples in molecular diagnosis

minimize invasiveness, achieving the best diagnostic outcome often requires selecting the specimen that provides the highest likelihood of detecting the pathogen. This chapter outlines the advantages and limitations of various sample types, providing clinical examples that illustrate their suitability in molecular diagnosis of viral diseases.

3.2 Blood as Most Common Choice of Sample for Molecular Diagnosis

Blood is a specialized connective tissue (Batra et al. 2022), in which various cellular elements—such as erythrocytes (red blood cells), leucocytes (white blood cells), and platelets—are suspended within the plasma (Zainab). Each playing distinct roles in the body's response to infections, including viral diseases. Blood cells

account for approximately 45% of the total blood volume, while plasma makes up about 55%. Plasma is a pale, straw-colored liquid, with water making up 91.5% of its volume. The remaining 8.5% consists of proteins (7%) and other solutes (1.5%). Plasma contains proteins such as albumin, globulin, fibrinogen, prothrombin, and heparin. Additionally, plasma contains antibodies, anti-toxins, hormones, and enzymes, all of which are essential for immune defense. Blood cells, which include erythrocytes (red blood cells), leucocytes (white blood cells), and thrombocytes (platelets), are vital for immune function and play a critical role in defending the body against viral infections. Leucocytes are particularly relevant for viral molecular diagnostics because they can carry viral genetic material (RNA or DNA) and are central to the immune response (Farley 2005). The analysis of these blood components, especially leucocytes, is crucial in detecting viruses and their genetic material, making them essential in molecular diagnostic techniques like PCR for viral identification.

Venipuncture, a common method for the collection of blood, involves inserting a needle into a vein, typically at the antecubital fossa, to collect a blood sample. This method is preferred when large volume of blood is needed. In contrast, capillary puncture is performed when venipuncture is difficult or when only small volume of blood is required, often for point-of-care testing. The blood collected in capillary puncture is a mixture of capillary, venous, and arterial blood, along with some tissue fluid, leading to potential variations in laboratory results compared to venipuncture samples, particularly for parameters like glucose, hemoglobin, hematocrit, pH, calcium, and magnesium (Lew n.d.).

3.2.1 Role of Anticoagulants

Anticoagulants play a vital role in blood sample preparation. Their primary functions include preventing blood clotting, maintaining sample stability, and preserving cellular or molecular integrity (Banfi et al. 2007). Commonly used anticoagulants such as sodium citrate, heparin, and ethylenediaminetetraacetic acid (EDTA) are added to blood collection tubes to block the clotting process. This action keeps the blood in a liquid state, which is essential for obtaining specific components such as plasma or white blood cells (leukocytes). Plasma, the liquid portion that remains after centrifugation, contains active clotting factors, setting it apart from serum. The anticoagulants prevent clot formation, allowing for the successful isolation of plasma, which is important for detecting biomarkers like antibodies or viral antigens circulating in the blood. Therefore, choosing the right anticoagulant is crucial for preserving the integrity of the sample, ensuring accurate and reliable diagnostic results (Louten 2016). Table 3.1 summarizes different types of anticoagulants used for various diagnostic blood tests.

Table 3.1 Summary of various anticoagulants and their suitability for various molecular and non-molecular diagnostic approaches along with universal identification color-code on the cap

Anticoagulant	Mode of action	Commonly used for	Color of collection tube (standard)
Ethylenediaminetetraacetic acid (EDTA)	Chelates calcium (Ca^{2+}), preventing clotting	Hematology (complete blood count—CBC, peripheral smear), molecular tests (DNA/RNA studies)	Purple/lavender
Sodium citrate	Binds calcium (reversible)	Coagulation studies (prothrombin time—PT, activated partial thromboplastin time—APTT, D-dimer)	Light blue
Heparin (lithium, sodium, or ammonium heparin)	Inhibits thrombin and other clotting factors	Blood gas analysis, chemistry tests (electrolytes, enzymes, hormones), flow cytometry	Green
Sodium fluoride + potassium oxalate	Sodium fluoride inhibits glycolysis; potassium oxalate precipitates calcium	Glucose estimation, lactate estimation	Gray
Acid citrate dextrose (ACD)	Binds calcium; dextrose preserves RBCs	Blood banking (HLA typing, DNA analysis), platelet function studies	Yellow
Citrate–phosphate–dextrose (CPD)	Preserves RBCs and prevents clotting	Blood transfusion, blood storage	Yellow
Sodium polyanetholesulfonate (SPS)	Inhibits complement and phagocytosis	Blood culture (microbial studies)	Yellow

3.2.2 Advantages

Blood offers distinct advantages as a choice sample in the molecular diagnosis of viral diseases, primarily due to its superior sensitivity and reliability when compared to alternative sample types. For chikungunya virus (CHIKV), reports suggest that serum samples offer the highest diagnostic sensitivity, particularly within the first five days of symptom onset (Jacob-Nascimento et al. 2024). In contrast, RT-qPCR in oral fluids showed only 50% of the sensitivity observed in serum, while urine samples exhibited an even lower sensitivity of 5%. These findings emphasize the critical role of blood-derived samples for accurate CHIKV diagnosis in clinical settings, especially during the acute phase of infection. Although oral fluids may serve as a convenient alternative for surveillance purposes, they fall short in providing definitive diagnostic accuracy. Similarly, Andries et al. (2015) demonstrated the diagnostic superiority of plasma for dengue virus detection. Plasma samples showed

the highest sensitivity, with 85.4% for RT-PCR and 63.4% for NS1 antigen detection. In comparison, urine samples achieved sensitivities of 41.6% for RT-PCR and 14.5% for NS1 antigen, while saliva samples performed worse, with sensitivities of 39% for RT-PCR and 28.3% for NS1. Even when urine and saliva samples were collected concurrently, the combined sensitivity for RNA detection (69.1%) and NS1 antigen detection (34.4%) remained inferior to plasma. These findings underscore the reliability of plasma samples for accurate dengue diagnosis while highlighting the limited utility of urine and saliva as complementary diagnostic tools. Collectively, these studies affirm the diagnostic advantage of blood samples for viral infections, making them indispensable in clinical diagnostics.

Given the clear diagnostic superiority of blood samples, the selection of blood preparation is also crucial, as different types of blood samples provide distinct advantages depending on the specific disease and diagnostic requirements. Studies have demonstrated that whole blood, serum, capillary blood, and plasma each have specific diagnostic utilities depending on the pathogen. In a study by Klungthong et al. (2007), they compared the effectiveness of reverse transcriptase PCR (RT-PCR) using whole blood and serum samples for detecting dengue virus infections in clinically suspected individuals. They found that RNA extraction from whole blood followed by RT-PCR resulted in a higher detection rate than serum or plasma samples. This is particularly advantageous during the acute phase of the disease, where viral RNA is more prevalent in whole blood. Similarly, other reports suggest that capillary blood, when stored on filter paper, demonstrated high sensitivity (81.6%) and specificity (90.7%) for detecting dengue virus via RT-PCR (Matheus et al. 2007). Additionally, capillary blood samples showed longer persistence of viral particles compared to venous blood. Moreover, capillary blood offers logistical benefits, such as ease of collection, no requirement for cold chain storage, and simplified sampling, which is crucial for improving diagnostic access in resource-limited settings.

In the context of HIV-1 viral load measurement, Mouna et al. (2023) demonstrated that serum could reliably replace plasma without significant differences in the results. The study found a strong correlation between serum and plasma measurements, even when viral loads were below 50 or above 400 copies/ml, which aligns with clinical thresholds. This suggests that serum is a practical alternative to plasma, particularly in urgent clinical situations where plasma might not be readily available, such as managing HIV in pregnant women or diagnosing primary infections. This flexibility can lead to faster treatment and improved patient care. Furthermore, some studies suggest that the whole blood samples exhibited 100% positivity for Epstein–Barr virus DNA, compared to only 32.1% in plasma samples (Rzepka et al. 2023). Whole blood also showed higher viral loads, which is critical in assessing the risk of reactivation and evaluating therapeutic efficacy, particularly in post-transplant or hemato-oncological patients. Together, these studies illustrate the importance of choice of sample and their preparation for maximizing the diagnostic sensitivity and improving patient outcomes.

3.2.3 *Limitations and Challenges*

While blood remains the preferred specimen for the molecular detection of many viral diseases, several challenges arise when comparing its diagnostic utility with other biological samples. Musso et al. (2016) showed that saliva and urine are not ideal replacements for blood for the detection of Chikungunya virus (CHIKV) RNA. Although saliva can serve as an alternative during the first week post-symptom onset if blood collection is not feasible; however, the sensitivity is significantly lower. Unlike Zika virus, where saliva and urine extend the detection window, these samples do not enhance CHIKV RNA detection.

Similarly, blood remains the preferred specimen for Dengue virus (DENV) and West Nile virus (WNV) RNA. Case studies highlight that alternative samples may offer diagnostic value under specific circumstances. For example, Mizuno et al. (2007) reported DENV RNA in urine and saliva but not in plasma, suggesting these as potential diagnostic samples. Likewise, Ma et al. (2014) detected DENV RNA in urine samples on days 8 and 18 post-symptom onset, even when serum samples tested negative, emphasizing the extended detection window that urine provides compared to serum.

Urine has also shown advantages in detecting WNV and ZIKV RNA. Nagy et al. (2016) reported that WNV RNA was detectable in urine for longer durations and at higher copy numbers compared to serum, particularly in patients with neurological symptoms. Barzon et al. (2013) corroborated these findings, demonstrating that urine consistently excreted higher titres of WNV RNA for longer periods than blood or cerebrospinal fluid (CSF), making it valuable for confirming WNV infection after viremia resolution. Gourinat et al. (2015) observed a similar trend for ZIKV, where RNA loads in urine remained higher and detectable for up to 15 days post-symptom onset, compared to serum samples that tested negative after 7 days.

These findings show that while blood is the primary choice for molecular diagnostics, alternative samples like urine and saliva can be useful in specific diseases.

3.3 Saliva as a Potential Non-invasive Sample for Molecular Diagnosis

Saliva is a complex fluid produced by the body, primarily secreted by three major salivary glands: the submandibular, parotid, and sublingual glands. However, it is not only these large glands that contribute to saliva production. Smaller glands found in the gingival folds and oral mucosa also secrete saliva, though in much smaller quantities. Human saliva is composed mostly of water (~98.5%), with the remaining 1.5% consisting of organic (1.0%) and inorganic (0.5%) components. The organic components includes—proteins and enzymes—amylases, lectins, peroxidase, lactoferrin, lysozyme, myeloperoxidase, secreted IgA, defensins, histone-rich protein, cysteine protease inhibitor, and mucin G1 and G2. Lipids—cholesterol,

fatty acids, diglycerides, triglycerides, neutral lipids, and glycolipids. Nitrogenous compounds include urea, uric acid, amino acids, and steroid hormones. The inorganic components of saliva are primarily electrolytes, along with significant amounts of trace elements such as Na^+, Cl^-, K^+, HCO_3^-, $H_2PO_4^-$, F^-, I^-, and Mg^{2+}, all of which contribute to the fluid's overall composition (Cui et al. 2022). The concentrations of these components in saliva have long been studied, as they can serve as valuable indicators or even auxiliary tools in diagnosing a variety of diseases. By analyzing changes in these components, important insights into a person's health can be gained, offering a non-invasive diagnostic approach (Dave et al. 2020).

There are four commonly used methods for saliva collection include the draining, spitting, suction, and swabbing techniques, each with distinct advantages and challenges. These methods are designed to accommodate various clinical needs, ensuring uncontaminated samples for molecular analysis. In contrast, the draining method involves the individual sitting with their head bent forward and mouth open, allowing saliva to naturally drip into a sterile container, resulting in an unstimulated sample. In the spitting method, saliva accumulates in the mouth and is then expelled into a pre-measured test tube. This method is effective for low saliva flow but may induce slight stimulation of the salivary glands. The suction method utilizes instruments such as micropipettes or saliva ejectors to aspirate saliva, providing precise control over volume and flow, making it suitable for accurate collection. The swabbing method uses a sponge or swab to absorb saliva from the mouth, primarily for monitoring oral conditions or specific substances, though it is not ideal for collecting unstimulated saliva (*Methods of Collection of Saliva—A Review—Int J Oral Health Dent*, n.d.; Bellagambi et al. 2020).

3.3.1 Advantage

Saliva is rapidly emerging as a valuable diagnostic sample for viral diseases, offering a combination of practicality, reliability, and broad diagnostic potential. Its unique characteristics—ease of collection, non-invasiveness make it an attractive alternative to traditional diagnostic specimens like blood or tissue. Furthermore, saliva can detect a wide range of viral markers, including DNA, RNA, antigens, and antibodies, expanding its utility across various viral infections.

Several studies have highlighted the potential of saliva as a reliable diagnostic sample for various viral infections. Wyllie et al. (2020) compared the sensitivity of saliva specimens and nasopharyngeal swabs in detecting SARS-CoV-2 in patients with COVID-19. Their findings indicated that saliva samples exhibited comparable sensitivity to nasopharyngeal swabs, with 81% of saliva samples testing positive compared to 71% of nasopharyngeal swabs within 1–5 days of diagnosis. Saliva samples showed less variation in SARS-CoV-2 RNA levels and fewer false-negative results, supporting the use of saliva as a diagnostic sample for COVID-19 testing. Similarly, Musso et al. (2015) found that Zika virus (ZIKV) RNA was more frequently detected in saliva than in blood, particularly during the acute phase of

infection. Among 182 patients, 19.2% were positive for ZIKV RNA in saliva but negative in blood, further highlighting saliva's role in viral detection.

In addition, saliva has also been explored as a diagnostic medium for other diseases. Tang et al. (2019) demonstrated that HPV-16 detection in saliva could serve as a biomarker for oropharyngeal cancer (OPC). The study showed a significant correlation between HPV-16 viral load and advanced OPC stages, supporting the use of saliva-based HPV-16 detection as a potential screening tool for at-risk individuals. Similarly, Victória et al. (2005) developed nested PCR to detect herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), and human herpesvirus 6 (HHV-6) in oral swabs, proving the reliability of saliva for viral identification. Saliva's diagnostic value extends to other viruses such as Puumala virus (PUUV) and HIV-1. Pettersson et al. (2008) found PUUV RNA detected in saliva from hospitalized patients, demonstrating its potential as a source for hantavirus detection.

Shugars et al. (2001) explored the potential of the oral cavity as an HIV-1 reservoir by analyzing viral loads in blood plasma and saliva. Their findings revealed HIV-1 RNA in 79% of plasma samples, 44% of unfiltered saliva, 16% of filtered saliva, and 59% of saliva-derived cell pellets. Notably, five individuals displayed salivary viral hyper-excretion, where viral load in saliva surpassed those in plasma.

Saliva is particularly useful in situations where blood collection is difficult, such as with children and neonates (Gorchakov et al. 2019). Given the promising findings across different viruses like SARS-CoV-2, HPV-16, ZIKV, and HIV-1, makes saliva an attractive alternative sample choice. With continued improvements in collection methods and assay protocols, saliva-based diagnostics may become a routine part of clinical practice, improving accessibility and reducing the burden on healthcare systems.

3.3.2 Limitations and Challenges

Saliva has emerged as a promising specimen for diagnostic testing, yet several challenges remains. To et al. (2019) explored the feasibility of saliva for respiratory virus detection and found that saliva was less sensitive than nasopharyngeal aspirates (NPA). The study revealed that 9.2% of cases were positive in NPA but not in saliva. Although saliva demonstrated good specificity (100%) and acceptable sensitivity (90.8%), it was less sensitive due to lower viral loads in comparison to NPA samples. Despite these limitations, saliva offers significant advantages, including non-invasive collection, reduced time and cost, and the potential for self-collection. The study suggested that with advancements in automated molecular assays, saliva could overcome its sensitivity challenges and become a more viable diagnostic tool.

To enhance the reliability and sensitivity of saliva-based diagnostics, it is critical to address several key factors. These include refining RNA detection methods, optimizing saliva collection techniques, and developing optimal solutions for sample transportation and preservation. Additionally, ensuring the inclusion of appropriate internal controls (Czumbel et al. 2020). Standardizing these parameters will improve the overall performance and diagnostic accuracy of saliva as a viable diagnostic tool.

3.4 Urine as a Sample for Molecular Diagnosis

Urine primarily consists of water, which acts as a solvent for various organic and inorganic compounds. Notable organic components include urea, creatinine, and uric acid. In healthy individuals, proteins account for a small fraction, with Tamm–Horsfall protein (uro-modulin) being the most abundant (50%), followed by albumin (20%) and immunoglobulins (5%). Additionally, it may contain trace amounts of proteins like bilirubin and urobilinogen, as well as cellular elements, including erythrocytes, leukocytes (Aitekenov et al. 2021; Queremel Milani and Jialal 2024). Urine collection is commonly done using the "clean-catch" method to minimize contamination. After cleaning the urethral area, patients void a small initial portion of urine and collect a mid-stream sample (15–30 mL) in a sterile container, which is then securely closed and labeled (Wu 2010).

Invasive urine collection methods are used when non-invasive techniques are not feasible, such as in cases of urinary incontinence or external urethral ulcers. One common approach is urethral catheterization, which involves the insertion of a small catheter into the urethra after proper cleaning. However, it is crucial to avoid collecting samples from a catheter bag, as this may lead to contamination. Suprapubic needle aspiration is a more invasive procedure typically used when contamination persists despite other methods. This technique involves the insertion of a needle into the bladder to collect urine directly (Queremel Milani and Jialal 2024).

3.4.1 Advantages

Urine has proven to be an effective alternative sample for various viral infections, offering several advantages over traditional blood-based testing. Lamb et al. (2018) developed an RT-LAMP-based diagnostic test for Zika virus (ZIKV) in urine, which provided comparable sensitivity to conventional qRT-PCR in a faster and more cost-effective manner, eliminating the need for RNA extraction. This makes urine a highly efficient sample choice for ZIKV monitoring, particularly in resource-limited settings. Similarly, Lu et al. (2019) demonstrated that urine could be used for detecting hepatitis C virus (HCV) RNA, with a real-time PCR assay significantly improving detection rate. The study also found a strong positive correlation between plasma and urine HCV RNA concentrations, suggesting urine's potential for non-invasive therapeutic monitoring in patients with HCV.

Urine has also shown promise in the detection of other viruses. Sari et al. (2020) highlighted the utility of RT-PCR in detecting measles virus in urine, proving more sensitive than viral culture, particularly when blood samples are not available. Reusken et al. (2017) detected yellow fever virus (YFV) RNA in urine samples up to 24 days post-symptom onset, emphasizing urine's extended window for viral detection. In the context of dengue virus (DENV), studies by Poloni et al. (2010) and Hirayama et al. (2012) illustrated that urine could detect DENV RNA early in

infection, with detection rates increasing as the disease progressed. Korhonen et al. (2014) further highlighted urine's extended detection window, with higher sensitivity for DENV RNA detection compared to serum and saliva, particularly between days 7 and 13 post-symptom onset.

As previously discussed in this chapter, studies by Nagy et al. (2016) and Barzon et al. (2013) demonstrated that urine provided a longer detection window and higher viral titres than serum in the case of West Nile virus (WNV), even after viremia had resolved. These findings collectively support the growing role of urine as a non-invasive, reliable, and extended diagnostic specimen for multiple viral infections, highlighting its potential for both early detection and prolonged monitoring.

3.4.2 Limitations and Challenges

Urine samples, despite their non-invasive nature, may not always be the ideal choice for viral diagnostics, with several studies highlighting limitations in sensitivity for certain viral infections. The effectiveness of urine as a diagnostic sample can vary significantly depending on the specific viral infection, as highlighted by several studies. Jacob-Nascimento et al. (2024) found that urine is not suitable for the diagnosis of Chikungunya virus (CHIKV). In their study, serum samples exhibited the highest diagnostic sensitivity for CHIKV, whereas urine samples showed a significantly lower sensitivity of only 5%. This highlights the challenge of selecting the appropriate sample type for molecular diagnostics based on the virus and clinical context. Similarly, Payan et al. (2007) found that while human papillomavirus (HPV) DNA was detected in 45% of cervical specimens, only 37% of urine samples tested positive. Moreover, the viral load in urine was 50 times lower than in cervical specimens, making urine a less reliable alternative for HPV screening. This underscores the challenge of achieving the same diagnostic sensitivity with urine samples as with more conventional specimen types. Additionally, a study by Wang et al. (2020) found no SARS-CoV-2 detection in urine samples from 205 COVID-19 patients, suggesting that urine may not be a viable sample for detecting the virus in COVID-19 cases. These findings illustrate the limitations of using urine for certain infections and emphasize the importance of selecting the most appropriate sample type to ensure accurate diagnostic outcomes.

3.5 Feces as Sample for Molecular Diagnosis

Feces is a complex mixture consisting of undigested fibers, intestinal secretions, nonabsorbable toxins, water, microbial particles, proteins, carbohydrates, nitrogenous compounds, fats, lipids, and various metabolites (Ali et al. 2022). Proper collection and preservation of fecal samples are essential to maintain their integrity for molecular and diagnostic analyses. Common methods include immersion in

stabilizing solutions such as Tris-EDTA buffer, RNAlater, or ethanol, which help stabilize nucleic acids and proteins. Commercial preservation tubes, such as OMNIgene.GUT and the Stool Nucleic Acid Collection and Preservation Tube (Norgen BioTek Corp), offer convenience and effectiveness in maintaining sample quality during transport and storage. Immediate freezing at −20 °C (fresh frozen) or −80 °C is another reliable method for preserving the structural and biochemical components of feces (Watson et al. 2019).

3.5.1 Advantages

Fecal samples have shown significant potential as an alternative diagnostic tool for viral diseases, supported by several studies. Li et al. (2020) and Mesoraca et al. (2020) highlighted the role of fecal samples for SARS-CoV-2 detection, especially when respiratory samples are inconclusive. Li et al. (2020) reported a case where a baby with mild COVID-19 tested positive for SARS-CoV-2 RNA in anal swabs, despite negative throat swabs and normal chest computed tomography (CT) results, indicating that fecal samples could be a valuable diagnostic alternative. Similarly, Mesoraca et al. (2020) observed that fecal specimens often remain positive for SARS-CoV-2 RNA longer than respiratory tract samples, underscoring their utility in cases where respiratory samples fail to yield conclusive results.

In gastroenteritis diagnostics, Kowada et al. (2018) and Khamrin et al. (2011) developed multiplex PCR assays to detect a wide range of viral pathogens in stool samples, including rotavirus, adenovirus, norovirus, and enterovirus, proving the versatility of fecal samples in detecting multiple viruses. Jeulin et al. (2011) also highlighted the diagnostic value of quantifying adenovirus DNA in stool samples for early detection, revealing that stool samples can provide earlier detection of adenoviraemia than whole blood, with a sensitivity of 89%. Additionally, Ayele et al. (2009) demonstrated that HIV-1 RNA could be detected in stool samples from infants, further supporting the potential of fecal samples in diagnosing viral infections in vulnerable populations. Kupila et al. (2005) found that PCR testing of fecal specimens for enteroviral meningitis showed a higher diagnostic sensitivity (96%) compared to cerebrospinal fluid (CSF) samples (76%), suggesting that fecal specimens should be a standard diagnostic tool in such cases. Overall, these studies emphasize the crucial role of fecal samples in diagnostics, particularly when other sample types may be inconclusive.

3.5.2 Limitations and Challenges

Fecal samples, while useful for molecular diagnosis, have several limitations that can affect the accuracy and reliability of results. One major challenge is the presence of PCR inhibitors, such as bile salts, complex polysaccharides, and heme

compounds, which can interfere with nucleic acid amplification, leading to false-negative results. Additionally, the degradation of nucleic acids due to digestive enzymes, microbial activity, and environmental factors can compromise the quality and yield of DNA or RNA, making detection difficult. The variability in sample composition, influenced by diet, hydration, and gut microbiota, can introduce inconsistencies in analysis, reducing reproducibility. Contamination risks are also high, as fecal samples contain diverse microbial populations, increasing the chance of cross-reactivity and misidentification of target pathogens. Furthermore, the uneven distribution of pathogens or biomarkers within the sample can lead to sampling bias, necessitating proper homogenization and multiple extractions for accurate results. Storage and transportation conditions also impact molecular stability, as prolonged exposure to heat and moisture can degrade nucleic acids, requiring immediate preservation methods like freezing or specialized transport media. Lastly, the presence of host DNA in fecal samples may complicate pathogen detection in metagenomic studies, necessitating optimized extraction protocols to enrich microbial DNA while minimizing host-derived contaminants.

3.6 Other Potential Samples

In addition to commonly used diagnostic samples, various other biological fluids have been explored for the presence of viral nucleic acid. Several studies have explored the presence of viral RNA in tear samples, offering valuable insights into their potential role in viral diagnostics. Qian et al. (2020) investigated HIV-1 viral load in tears and found detectable HIV-1 RNA in 53.3% of untreated HIV/AIDS patients. Viral loads in tears were lower than those in plasma, and a correlation with plasma viral load and AIDS stage was observed. Regarding SARS-CoV-2, studies have examined its presence in tears during the early symptomatic stage. Sonmez et al. (2022) found that only 2.5% of tear samples tested positive for SARS-CoV-2, with viral loads significantly lower in tears than in naso-oropharyngeal samples. Similarly, Sabage et al. (2022) detected SARS-CoV-2 in 18.2% of conjunctival swab (CS) samples and 12.1% of Schirmer strip (SS) samples from hospitalized patients with COVID-19. These findings suggest that while tear samples may provide some diagnostic information, the lower viral loads in tears compared to plasma or naso-oropharyngeal samples may limit their clinical utility.

Hoarau et al. (2023) evaluated real-time quantitative PCR (RT-qPCR) for diagnosing herpes simplex keratitis (HSK) using tear samples. This study, which reviewed clinical records of 364 patients, found that RT-qPCR for herpes simplex virus type 1 (HSV-1) had a sensitivity of 31.6% and specificity of 99.5%. While RT-qPCR of tear samples is useful for confirming HSK diagnosis, especially in cases with epithelial involvement, its sensitivity may vary across different forms of the diseases. In the context of West Nile virus (WNV), Briese et al. (2000) and Lanciotti et al. (2000) contributed to improving the diagnosis of WNV in cerebrospinal fluid (CSF), aiding in viral encephalitis diagnosis. Briese et al. (2000)

established a real-time PCR method to detect WNV sequences in CSF, while Lanciotti et al. (2000) developed a rapid TaqMan assay for the same purpose. The TaqMan assay demonstrated greater sensitivity compared to traditional RT-PCR and virus isolation methods, accurately identifying WNV in 98% of culture-positive CSF samples. These advancements underscore the importance of molecular diagnostic methods for the rapid and accurate detection of WNV in CSF.

Recent studies have also highlighted the presence of viral nucleic acids in amniotic fluid (AF). Gervasi et al. (2012) detected viral nucleic acids in 2.2% of AF samples from asymptomatic women in the mid-trimester, with human herpesvirus 6 (HHV-6) being the most common. Other detected viruses included human cytomegalovirus (HCMV) and parvovirus B19, although HSV, VZV, enteroviruses, and adenoviruses were not found. Calvet et al. (2016) reported detecting Zika virus in the amniotic fluid of two pregnant women, although no virus was found in their urine or serum. Overall, these studies highlight the evolving landscape of viral diagnostics, where unconventional samples may provide additional insights into disease detection and monitoring. However, further research is needed to optimize their diagnostic value and applicability in clinical settings.

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Chapter 4
Molecular and Serological Approaches for the Diagnosis of Human *Orthoflaviviruses*

Ayesha Tazeen, Nazim Khan, Abdus Samad, Sonam Gupta, Anwar Ahmed, and Shama Parveen

Abstract The infection caused by *Orthoflaviviruses* is a global health concern and causes millions of infections and economical loss every year. Pathogenic *Orthoflavivirus* infecting human includes the Dengue virus (DENV), Japanese encephalitis virus (JEV), Zika virus (ZIKV), West Nile virus (WNV) and Yellow fever virus (YFV) causing disease ranging from mild fever to severe manifestations including hemorrhagic fever and encephalitis. These *Orthoflaviviruses* causes similar symptoms and often co-infect and co-circulates in endemic areas which sometimes lead to misdiagnosis. Therefore, accurate and early diagnoses of *Orthoflavivirus* infections are inevitable. The current chapter describes the molecular and serological diagnostic techniques which are currently in use such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), isothermal techniques like nucleic acid sequence-based amplification (NASBA), reverse transcription loop-mediated isothermal amplification (RT-LAMP) including others. These diagnostic methods were explored for their ease of use, minimum time involved in the assay, specificity, sensitivity, low cross-reactivity and false-positive results. The rapidity and sensitivity of techniques for differential diagnosis of *Orthoflaviviruses* is essential for proper disease management and thus improvements in the current techniques are needed for effective patient management.

Keywords *Orthoflaviviruses* · Diagnosis · Molecular techniques · Serological detection · Isothermal assays

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4.1 Introduction

The diagnosis of arthropod-borne viral infections is an essential field of research due to their significant impact on the global health. The virus of genus *Orthoflavivirus* infecting humans includes Dengue virus (DENV), Yellow fever virus (YFV), Zika virus (ZIKV), West Nile virus (WNV), and Japanese encephalitis virus (JEV). The emergence and re-emergence of these *Orthoflaviviruses* in the last few decades caused several epidemics, co-morbidities, significant impact on the world economy, loss of healthcare resources and severe diseases leading to death. The *Orthoflavivirus* infections show similar clinical symptoms and majority of the infected hosts are either asymptomatic or have mild fever and thus are often misdiagnosed at early stage. Thus, the diagnosis of *Orthoflavivirus* infections based only on the signs and symptoms is unreliable. The diagnosis is more challenging in areas where these *Orthoflaviviruses* and other *Arboviruses* such as Chikungunya and Malaria co-circulates and co-infects (Tazeen et al. 2017a, b; Abdullah et al. 2017; Hisamuddin et al. 2018; Alves and Amorim 2023). Therefore, specific diagnostic techniques are essential for distinguishing *Orthoflavivirus* infections from other febrile diseases for effective patient management especially during epidemics in areas where co-circulation is frequently reported (Mori et al. 2021).

Early and accurate diagnosis will help to prevent unnecessary disease complications, treatment and sometimes hospitalisations. For instance, studies have reported that implementing early fluid treatment in dengue fever patients will help to prevent disease severity and hemorrhagic fever (World Health Organization 2011; Hung 2012). Further, prompt diagnosis and early supportive treatment have also minimised the long-term neurological damage in patients infected with WNV (Sejvar et al. 2003; Leis and Stokic 2012). Timely diagnosis and administration of potential antiviral treatments and use of interferon-alpha has been proven to reduce the disease severity in JEV infected patients (Joe et al. 2022). The differential diagnosis will also help to avoid unnecessary treatments and medications which may not be necessary for these self-limiting *Orthoflavivirus* infections especially in areas where co-circulation of febrile illness is common (Musso and Desprès 2020). A retrospective review showed the unnecessary use of antibiotics in hospitalised children in Indonesia infected with DENV but suspected of having bacterial infections (Adrizain et al. 2019). It highlights the importance of implementing rapid and accurate diagnosis and to decrease the occurrence of antibiotic resistance (Oidtman et al. 2021; Anyanwu and Duru 2023).

Apart from providing treatment, the diagnostic data also improves epidemiological surveillance and helps the public health officials to monitor disease patterns, identify new outbreaks, and determine the efficiency of available preventive measures. For instance, the ZIKV diagnostics played a significant role for understanding the extent and consequences of the 2015–2016 outbreak, especially in case of congenital Zika syndrome (Oidtman et al. 2021). During the 2015–2017, ZIKV

epidemic, a study calculated that there were 74,200 cases (based on clinical presentation with or without laboratory confirmation) of ZIKV infection in the last quarter of 2015 which were mistakenly identified as either Dengue or Chikungunya fever. With the increase in the number of reported ZIKV infection, the frequency of misdiagnosis increased in 2016 which varied by country depending on increased ZIKV infection cases as well as the laboratory testing method used. The disparities between serological and molecular diagnosis was particularly evident when the ZIKV pandemic was at its peak, indicating the significantly lower specificities and sensitivities associated with serological diagnostics (i.e., increased likelihood of misdiagnosis) in comparison to molecular diagnostics (Oidtman et al. 2021).

4.2 Diagnosis of *Orthoflavivirus* Infections

The advent of molecular biology technologies in the 1980s revolutionised the laboratory diagnosis of viral infections. Traditional approaches, such as viral isolation in cultured cells that may take several days to weeks have been replaced by molecular detection of virus genomes, which can be performed in hours. As discussed above, relying only on the clinical presentation is unreliable for identifying infection and thus lead to inappropriate or delayed treatment which may sometimes lead to severe cases. Thus, timely and specific diagnosis of viral infection is a necessity. In the acute phase of viral infection (1–7 days after the onset of symptoms), viral RNA, IgM, IgG and NS1 protein can be detected by different molecular and serological methods. The viral RNA and NS1 protein secreted during virus replication, which are critical indicators of viremia, are generally detected using the blood, plasma, and serum streams (Reta et al. 2020; Dias et al. 2023). In some cases of *Orthoflavivirus* infection, the viral RNA can also be found in the saliva, semen, urine, and vaginal fluids (Vina-Rodriguez et al. 2017; Cunha et al. 2020; Roldán et al. 2021; Daidoji et al. 2021; Dias et al. 2023). Also, the IgG antibodies can be detected for several months to years and is significant during vaccine trails, seroprevalence studies and previous infection studies especially in case Dengue fever in which secondary infection with different serotypes leads to severe dengue or hemorrhagic fever due to antibody-dependent enhancement (ADE).

Currently, several molecular and serological diagnostic methods are available for *Orthoflavivirus* detection. Apart from the conventional techniques several other diagnostic methods using new tools such as biosensors, nanotechnology and CRISPR 12/13 assay, lab on chip (LOC) including others are described in the literature. However, these new techniques are currently under development and far from being cost effective and reaching diagnostic laboratories and public. Thus, this chapter only describes the current understanding of the conventional and some new molecular and serological diagnostic methods for detecting infections caused by *Orthoflaviviruses* including DENV, WNV, YFV, ZIKV and JEV.

4.3 Diagnostic Techniques

4.3.1 Virus Culture and Isolation

The direct detection of pathogens is crucial in the diagnosis of *Orthoflavivirus* infection and vaccine studies. In the 1930s, the YFV is grown on the cell line, which aimed for vaccine production followed by first virus isolation in 1950s (Lloyd et al. 1936; Hematian et al. 2016). Virus isolation is most effective when patient samples are collected in the viraemic phase, i.e., 2–3 days before fever onset, and continues for 2–3 days (Gyawali and Taylor-Robinson 2017). Typically, serum is the preferred sample choice, but plasma, cerebral fluid, pleural fluid and peripheral blood can also be used. Additionally, tissues from the reticuloendothelial system, such as lymph nodes, spleen, liver, thymus, and lung, are occasionally used for virus isolation (WHO 2009; Hematian et al. 2016). The sensitivity of the virus isolation method relies on the time of sample collection, storage and transportation (Guzmán and Kourí 2004; Hematian et al. 2016; Baylis et al. 2021). The most used virus isolation method is by infecting insect cell line (C6/36 cell line) or mammalian cell line (Vero, Vero E6, LLCMK2, BHK-21 cell line) using patient sample. These cell lines have several different receptors such as DC-SIGN, Mannose-binding receptor (MR), heparan sulphate, Claudin-1 for DENV which initiate virus adsorption (Cruz-Oliveira et al. 2015). Briefly, the patient sample is collected in sterilised conditions and diluted using cell culture media followed by inoculation of monolayer cells (in 12 of 24 well plate or T25 cm^2 flask) and incubation at 37 °C for virus quantification (increase in titre). The cells are observed for 2–14 days (depends on serotype/genotype/strain of virus) or until cytopathic effect (CPE) is observed. The CPE refers to the change in the morphology of the infected cells including shrinking, swelling and syncytium formation confirming the presence of viral infection (Fig. 4.1). Further, the cell culture supernatant (having virus) is separated by centrifugation and confirm the identity of the isolated virus (Hematian et al. 2016; Baylis et al. 2021). Additionally, the virus isolation and detection can be also be done by injecting specimens intracerebrally into mice, inoculating larval or adult mosquitoes with specimens (Gyawali and Taylor-Robinson 2017; Piantadosi and Kanjilal 2020).

Earlier virus isolation method was the most widely used technique and was regarded as the specific gold standard for detecting RNA viruses, especially Dengue virus (Shu and Huang 2004; Peeling et al. 2010; Piantadosi and Kanjilal 2020). But due to its tedious, multi-step, high-cost and time-consuming protocol, its usage in diagnosis has been declined and is replaced by other molecular and serological methods. The major drawback is the requirement of biosafety laboratory (BSL) 2 or 3 or biocontainment facilities, regular maintenance of cell line, need of additional polymerase chain reaction (PCR) or immunoassay for identification of infecting virus and highly trained personnel.

Fig. 4.1 Virus culture and isolation. The patient serum is serially diluted followed by infection in preformed monolayer cells and incubated at 37 °C. The cells are observed for 2–14 days or until cytopathic effect (CPE; change in cells morphology such as syncytia formation, cell clumping or swelling) is observed. The presence of CPE confirms viral infection. For identifying virus, the supernatant (containing viral particles) is collected by centrifugation followed by PCR (using virus/serotype specific primers) or molecular or serological methods

4.3.2 Serological Diagnosis

Serological assays are frequently used for diagnosing viral infections due to their convenience compared to cell culture and RNA isolation. Distinct patterns of antibody responses are reported in the host with primary and subsequent secondary infection. In the *Orthoflavivirus* infection, the immunoglobulin M (IgM) antibodies are generally detectable from the first week to three months. The IgG antibodies can be detected after 2 weeks to months and sometimes even years. The host kinetics of antibody response against *Orthoflavivirus* may vary significantly depending on

several factors, such as the specific biological assay used, the viral antigens targeted for antibody capture and the host immune system. Also, the host immune system condition due to previous *Orthoflavivirus* exposure via vaccination or natural infection and the presence of primary and secondary *Orthoflavivirus*-related human infection are some of the major factors in serological diagnosis (Guzmán and Kourí 2004; Mansfield et al. 2011; Chong et al. 2019; Fischer et al. 2021). Commercial kits for serological diagnosis of DENV (MyBioSource, USA), YFV (Abbexa, UK) and ZIKV (Vircell, Spain), including others, are readily available in the market for rapid detection. However, as a result of potential cross-reactivity with closely related viruses, it is necessary to perform confirmatory tests, such as the plaque reduction neutralisation test (PRNT), which measures the levels of neutralising antibodies in serum or cerebrospinal fluid (CSF) samples (Fischer et al. 2021; Varghese et al. 2023).

Despite being comparatively rapid and cost-effective, serological assays have several limitations. False-positive results may arise due to the cross-reactivity between closely related viruses, including ZIKV, DENV, JEV, WNV and YFV (Chan et al. 2022). Additionally, individuals with weakened immune systems and reduced B-cell responses may experience inaccurate, false-negative serology results (Piantadosi and Kanjilal 2020). Also, in indirect assays, antibodies may not be detectable during the initial 3–5 days after infection, leading to the possibility of false-negative results at early-stage infection. Furthermore, these assays do not provide any information about the serotype of the virus; therefore, molecular techniques are imperative. Nonetheless, it is important to note that these assays might also have low sensitivity (Varghese et al. 2023). Some of the major serological methods for *Orthoflavivirus* detection are described below.

4.3.3 Plaque Reduction Neutralisation Test (PRNT)

The neutralising antibodies (nAbs) are necessary to provide immunity against *Orthoflavivirus* infecting humans in both vaccination trials and natural infection (Sabchareon et al. 2012; Bos et al. 2024). The measure and composition of pre-existing nAbs differ based on specific serotypes/genotypes and the number of past infections. PRNT is an essential assay used to measure the levels of nAbs and is now considered as the most reliable method for evaluating the neutralising capacity of nAbs (Thomas et al. 2009; Sabchareon et al. 2012; Ribeiro et al. 2020; World Health Organisation 2022; Dia et al. 2023; Bos et al. 2024).

The PRNT assay is performed using serum or plasma samples diluted in a series to determine the level of viral infectivity. To carry out the PRNT assay, a cell line (generally Vero cells are used) is cultured and seeded in a 12- or 24-well cell culture plate for monolayer formation. The complement proteins of the test samples are inactivated by heating them at 56 °C for approximately 30 min. Serially diluted infected serum samples are then incubated with the quantity of viral particles at 37 °C for one hour to facilitate interaction between viral particles and antibodies.

The virus–serum mixture is then loaded into the 24-well plate with a pre-formed cell monolayer and is incubated for an hour (may vary according to the different types of *Orthoflavivirus*) to achieve virus adsorption. The cells are then covered with carboxymethyl cellulose (CMC) or agarose diluted with cell culture maintenance media (DMEM, MEM) to limit the spread of the adsorb virus followed by incubation at 37 °C in CO_2 incubator for 3–7 days (based on the *Orthoflavivirus* being studied). After observing the cytopathic effect in the monolayer, cells are then washed with 3–5% formaldehyde, followed by staining using crystal violet or any other similar dye. The dye enables visualisation of plaques formed by viral infection and thus can be counted easily. The neutralisation titer is calculated using dilution at a point where there is a 50% decrease in plaque numbers compared to the viral control without serum (Fig. 4.2). This titer indicates the existence and extent of neutralising antibodies in the blood, offering valuable data for diagnosing *Orthoflavivirus* and evaluating immunological responses (Roehrig et al. 2008; World Health Organization 2009, 2022; Maeda and Maeda 2013; Piantadosi and Kanjilal 2020).

A recent study developed a PRNT assay against YFV which optimised various parameters such as virus-serum neutralisation time period and cell concentration. It evaluated the inter and intra assay precisions, specificity, and lower limit of quantification (LLOQ) using international standard yellow fever serum, specimens collected during YFV infection surveillance and sera from vaccine recipients (Dia et al. 2023). The assay demonstrated significant robustness and achieved 100% specificity, 100% precision in intra-assay, 95.6% in inter-assay, 95.3% dilatability and 100% LLOQ. Thus, this assay can be used in diagnosing YFV infection and conducting risk assessment studies by simultaneously performing PRNT for other *Orthoflavivirus,* especially where these viruses co-circulate (Dia et al. 2023). Further, this assay can evaluate the response of yellow fever vaccination nAbs or yellow fever seroconversions and vaccine efficacy in clinical trials, including fractional vaccine dose strategy (Dia et al. 2023). Similarly, another study developed a PRNT assay with significant diagnostic performance and showed the ability to differentiate between patients with and without a past DENV infection. PRNT and its modified versions have been widely used in DENV diagnosis, servo-prevalence studies, and in vaccine research using standard methods (Thomas et al. 2009; Mattia et al. 2011; Sasmono et al. 2018; Raafat et al. 2019; Ooi 2021; Limothai et al. 2021; Frazer and Norton 2024). For instance, PRNT has been actively used in clinical trials of Dengvaxia (CYD-TDV) and other developing vaccines against DENV to assess its effectiveness by measuring nAbs generated after immunisation against all the serotypes (Ooi 2021; Salje et al. 2021). This data is considered significant in determining the effectiveness of the vaccine. In a study conducted by Loyola et al., the ZIKV PRNT showed specificity and sensitivity of over 95%, regardless of prior DENV infection (Loyola et al. 2021). In the case of WNV and JEV, PRNT assays have also been developed but still exhibit some drawbacks similar to other *Orthoflavivirus* (Ledermann et al. 2011; De Filette et al. 2012; Hirota et al. 2013; Bharucha et al. 2020; Park et al. 2022; Nwanosike et al. 2023).

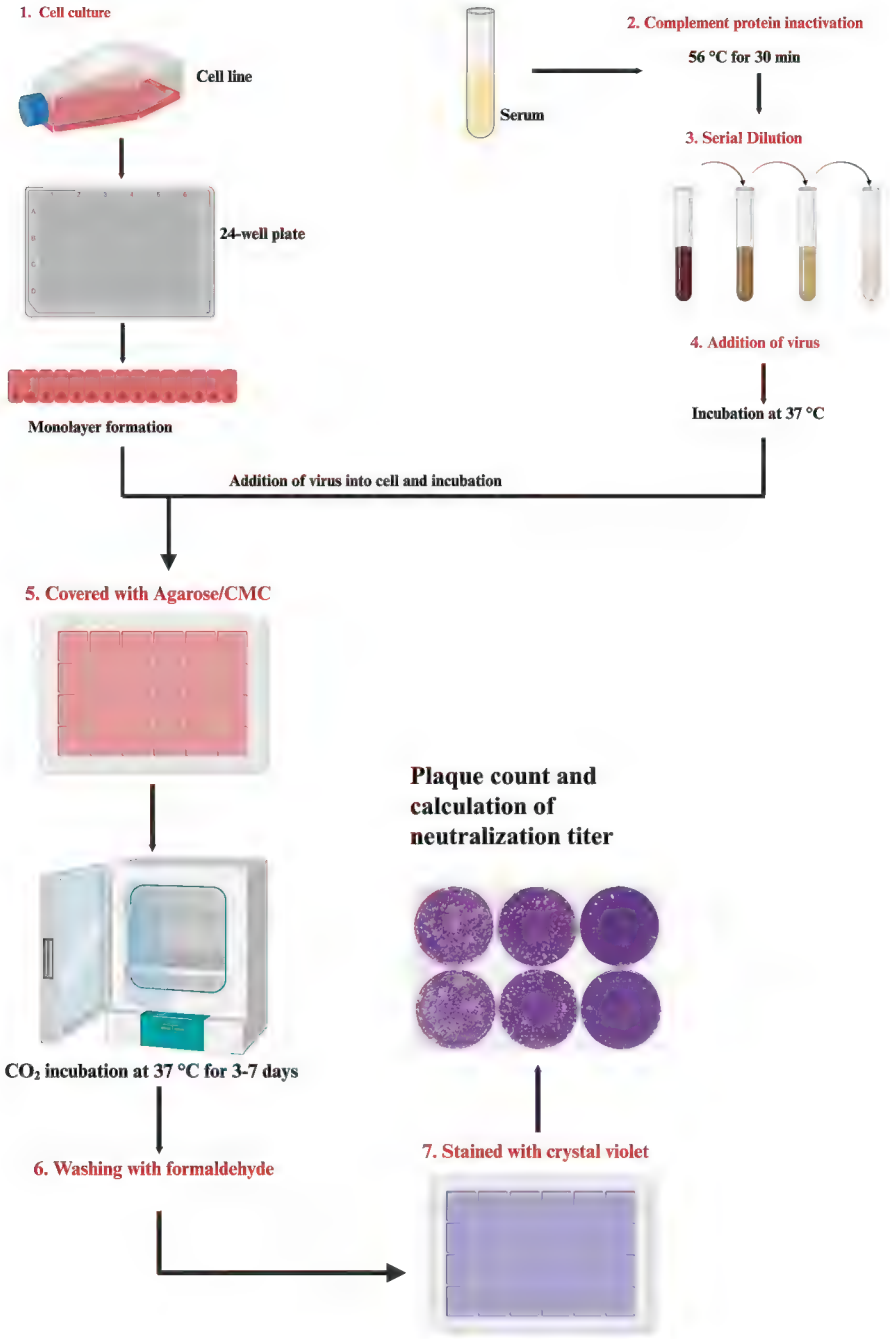
1. Cell culture
Cell line
24-well plate
Monolayer formation
2. Complement protein inactivation
56 °C for 30 min
Serum
3. Serial Dilution
4. Addition of virus
Incubation at 37 °C
Addition of virus into cell and incubation
5. Covered with Agarose/CMC
CO2 incubation at 37 °C for 3-7 days
6. Washing with formaldehyde
7. Stained with crystal violet
Plaque count and calculation of neutralization titer

Fig. 4.2 Plaque reduction neutralisation test (PRNT). The serum sample is serially diluted using cell culture media followed by infection of monolayer cells and incubation at 37 °C. After 1–2 hours of virus adsorption, the media is removed, and cell are covered with overlay media (carboxymethyl cellulose or agarose) diluted with DMEM/MEM and incubated at 37 °C for 3–7 days. The cells are fixed with 3–5% formaldehyde and stained using crystal violet dye. The plaque is manually counted

Although virus neutralisation assays are the reference tests for diagnosing *Orthoflavivirus* infection, serological cross-reactions between the *Orthoflavivirus* are also reported with PRNT, and variations in PRNT titres varied according to testing conditions (Roehrig et al. 2008; Ward et al. 2018; Musso and Desprès 2020). Additionally, even though PRNT is exceedingly specific and is unaffected by previous infection, it is seldom utilised in the laboratories or during epidemics for quick diagnosis as it requires highly skilled staff, is very expensive, requires BSL-2 or BSL-3 conditions and has high turnaround time (4–10 days). PRNT can determine a specific serotype of the virus during primary infection as it elicits a monotypic immune response. But in cases of secondary infection, the antibody response might be cross-reactive because of the previous infection and thus the serotype determined by PRNT is unreliable. Furthermore, several variations of PRNT assay are currently being used. Still, neither the assay nor its performance has been standardised between laboratories, and also there is no consensus among clinical laboratories on the optimum plaque reduction percentage (Peters and Stevenson 2019). However, recommendations for PRNT have been published to establish a standardised technique and interpretation of data. A study by Stephen et al. used late and acute convalescent follow-up serum samples from minor patients suffering from primary and secondary Dengue fever (Thomas et al. 2009). They evaluated PRNT to assess its performance under various conditions, including cell type, control virus passage, and complement use across multiple assays runs of the same sample panel. Their results showed wide variation in results and PRNT titer in response to varied testing conditions. The data emphasized the possible risks of comparing nAbs measurements between different laboratories, especially while evaluating the immunogenicity of viral vaccine candidates, and the need to harmonise and standardise the PRNT for each *Orthoflavivirus*. Nevertheless, although PRNT has its drawbacks, it is still considered as a gold standard confirmatory assay due its comparatively high specificity and sensitivity for detecting *Orthoflavivirus* infections and for quantifying nABs, especially during vaccine studies.

4.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

The antibodies generated during the *Orthoflavivirus* infection persist in the host for around two to three months, and in some instances, even longer (Chong et al., 2019; Kabir et al., 2021). The main difficulty faced by serological approaches that identify antibodies produced against *Orthoflavivirus* is that the samples collected before to the 5-day window may not yet show detectable levels of antibodies (Chao et al. 2015; Chong et al. 2019; Kabir et al. 2021). Initial or primary infections elicit a stronger IgM antibodies response, but subsequent or secondary infections provoke a stronger response from IgG antibodies. IgG antibodies are detectable one week after the onset of fever and decline after a few weeks, yet they can be detected after several years (Chao et al. 2015; Gyawali and Taylor-Robinson 2017; Chong et al. 2019). A significant four times increase in IgG antibodies is commonly associated

with a recent illness (Andries et al. 2012; Sharp et al. 2019). Currently, the IgM ELISA tests have become indispensable for monitoring DENV and other *Orthoflavivirus*. The sensitivity and accuracy of IgM-based test are significantly affected by the quality of antigen and display variation among commercially available kits. The study by Peeling et al., showed that IgM antibodies can be detected within 3–5 days after the onset of fever in approximately 50% of admitted patients with 90% sensitivity and 98% specificity (Peeling et al. 2010). ELISA can be performed in several ways such as direct, indirect, competitive, sandwich and multiplex based on the order in which antigens and antibodies are introduced in the assay (Aydin 2015). There are four basic steps in ELISA, i.e., coating (with either Ab or Ag), blocking (usually by adding bovine serum albumin; BSA) and detection followed by final calorimetric reading (Alhajj et al. 2024).

4.3.5 Direct ELISA

The direct ELISA was developed to identify specific antibodies against the human infecting *Orthoflavivirus*. The first step in the direct ELISA involves applying viral antigens diluted in a suitable coating buffer such as carbonate–bicarbonate buffer into each well of a microtiter plate (generally 96-well plate) overnight at 4 °C. This step provides a solid surface that can effectively bind antibodies from the samples. The selection of antigen and the conditions used for coating are crucial factors that determine the specificity and sensitivity of the assay. This step is followed by blocking non-specific binding sites with a solution such as 1–5% BSA or non-fat dry milk in PBS to prevent background interference. Patient serum samples are added to wells, allowing *Orthoflavivirus*-specific antibodies to bind directly to immobilised antigens. After incubation, plates are washed with a buffer like PBS containing Tween 20 to remove unbound antibodies and other contaminants. Following a second incubation and washing step, a substrate/chromogen solution such as 3,3′,5,5′-tetramethylbenzidine (TMB) is added, producing a colour change in the presence of the enzyme, horseradish peroxidase (HRP). No colour will be generated if there is no antigen/antibody present in the samples. The intensity of the colour is directly correlated with amount of antigen–antibody complex formed. This colour change can be quantified using a spectrophotometer or ELISA plate reader at 450 nm (Fig. 4.3). Results are then compared to control samples and predefined cut-off values to determine the presence of viral specific antibodies, indicating an infection (Pankaj 2013; Aydin 2015; Payne 2017; Silva et al. 2020; Alhajj et al. 2024).

4.3.6 Indirect ELISA

The indirect ELISA is generally used to detect antibodies but requires additional or secondary antibody which thus increases the assay cost. Like direct ELISA, the diluted antigens (in appropriate buffer) are fixed by overnight incubation at 4 °C

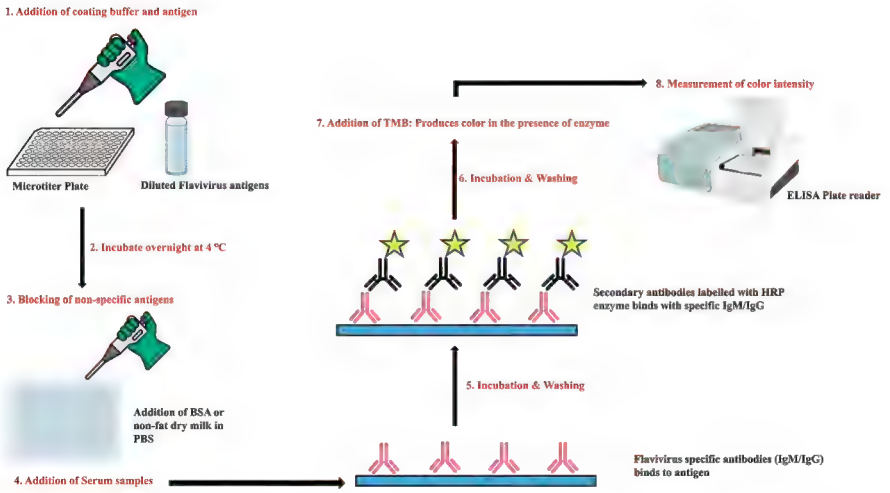
1. Addition of coating buffer and antigen
Microtiter Plate
Diluted Flavivirus antigens
2. Incubate overnight at 4 °C
3. Blocking of non-specific antigens
Addition of BSA or non-fat dry milk in PBS
4. Addition of Serum samples
Flavivirus specific antibodies (IgM/IgG) binds to antigen
5. Incubation & Washing
Secondary antibodies labelled with HRP enzyme binds with specific IgM/IgG
6. Incubation & Washing
7. Addition of TMB: Produces color in the presence of enzyme
8. Measurement of color intensity
ELISA Plate reader

Fig. 4.3 The ELISA includes four basic steps: coating, blocking, detection and reading. In direct ELISA, the serum sample (antigen) is coated and incubated on microtiter plate, followed by blocking of non-specific antigens using blocking buffer. The detection is done by adding enzyme conjugate primary antibodies and its substrate followed by final calorimetric reading using ELISA plate reader

onto a microtiter plate followed by blocking using buffer (BSA or non-fat dry milk) to inhibit non-specific binding. Diluted patient serum is loaded in the microtiter plate and incubated to allow the antibodies to bind to specific antigens fixed in the microtiter plate followed by washing. Next, an enzyme-labelled secondary antibody is added onto each well, specifically binding to human IgM or IgG. This secondary antibody is conjugated with an HRP enzyme. After incubation, extensive washing is done followed by addition of a substrate (such as TMB) leading to colorimetric shift in the samples, which is directly proportional to the quantity of antibody present (Fig. 4.4). The reaction is stopped by adding a solution (such as sulphuric acid) and quantified at 450 nm using an ELISA reader (Aydin 2015; Shah and Maghsoudlou 2016; Kohl and Ascoli 2017a; Alhajj et al. 2024).

4.3.7 Sandwich ELISA

The sandwich ELISA involves immobilising of capture or primary antibody in the microtiter plate that specifically targets the viral antigen. After blocking procedure, the sample containing the *Orthoflavivirus* antigen is added and incubated for attachment to capture antibody. After washing, an *Orthoflavivirus*-specific detection antibody is loaded which binds to another region or epitope of antigen. The detection antibody is conjugated with an enzyme, leading to calorimetric change after substrate addition (Fig. 4.5) (Shah and Maghsoudlou 2016; Tabatabaei and Ahmed 2022; Alhajj et al. 2024). The sandwich ELISA is highly useful due to its high

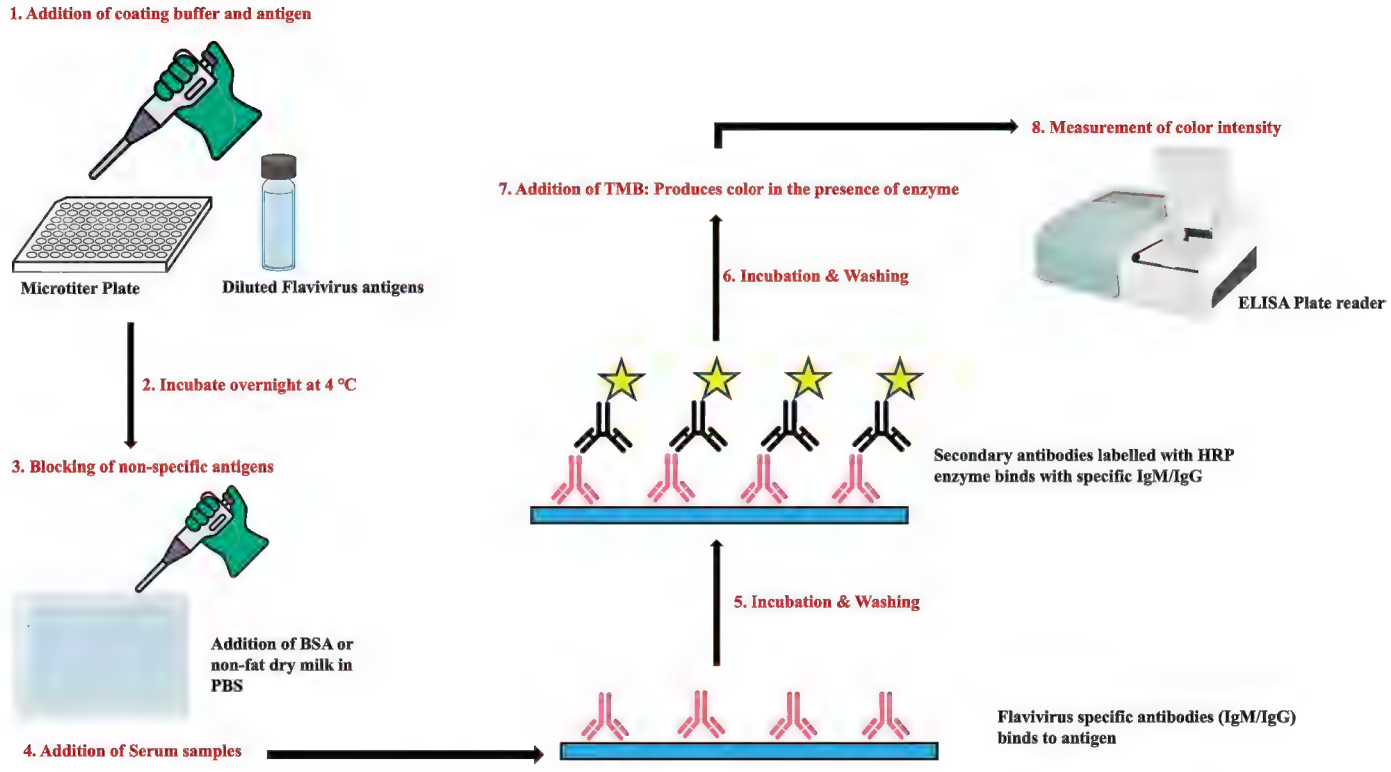
1. Addition of coating buffer and antigen
Microtiter Plate
Diluted Flavivirus antigens
2. Incubate overnight at 4 °C
3. Blocking of non-specific antigens
Addition of BSA or non-fat dry milk in PBS
4. Addition of Serum samples
Flavivirus specific antibodies (IgM/IgG) binds to antigen
5. Incubation & Washing
Secondary antibodies labelled with HRP enzyme binds with specific IgM/IgG
6. Incubation & Washing
7. Addition of TMB: Produces color in the presence of enzyme
8. Measurement of color intensity
ELISA Plate reader

Fig. 4.4 In indirect ELISA, after coating and blocking steps, the primary antibodies are added which bind to specific viral antigen present in the sample. Next, the enzyme bound secondary antibodies are added for detection which increases assay's sensitivity. Finally, the calorimetric readings are recorded using ELISA plate reader

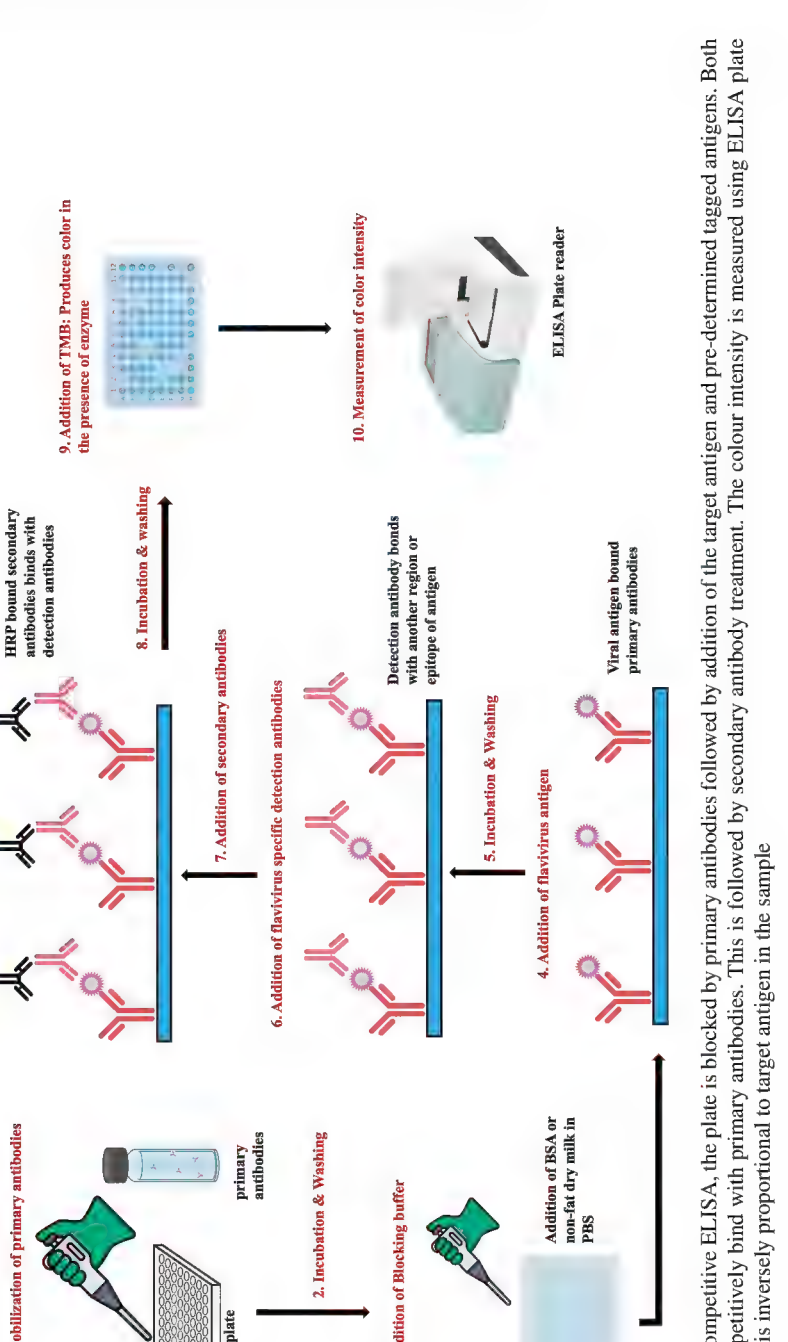
1. Immobilization of primary antibodies
Microtiter plate
primary antibodies
2. Incubation & Washing
3. Addition of Blocking buffer
Addition of BSA or non-fat dry milk in PBS
4. Addition of flavivirus antigen
Viral antigen bound primary antibodies
5. Incubation & Washing
Detection antibody bonds with another region or epitope of antigen
6. Addition of flavivirus specific detection antibodies
7. Addition of secondary antibodies
HRP bound secondary antibodies binds with detection antibodies
8. Incubation & washing
9. Addition of TMB: Produces color in the presence of enzyme
10. Measurement of color intensity
ELISA Plate reader

Fig. 4.5 In competitive ELISA, the plate is blocked by primary antibodies followed by addition of the target antigen and pre-determined tagged antigens. Both antigens competitively bind with primary antibodies. This is followed by secondary antibody treatment. The colour intensity is measured using ELISA plate reader which is inversely proportional to target antigen in the sample

specificity and capability to precisely detect very low concentration of antigen. This makes it extremely valuable for the early detection and monitoring of *Orthoflavivirus* infections (Vista et al. 2023).

4.3.8 Competitive ELISA

The procedure of competitive ELISA entails applying a layer of a particular antibody onto a microplate, which can attach to an *Orthoflavivirus* antigen. Next, the target antigen is introduced into the sample, along with a predetermined quantity of a tagged antigen that competes with the sample antigen for binding to the antibody. Following the incubation and washing steps, a detection is done by enzyme-linked secondary antibody that specifically binds to the labelled antigen, producing a quantifiable signal. The signal intensity is inversely proportional to the target antigen concentration in the sample, enabling accurate quantification (Fig. 4.6) (Aydin 2015; Kohl and Ascoli 2017b; Tabatabaei and Ahmed 2022).

Direct ELISAs are characterised by poor sensitivity, while competitive and indirect ELISAs exhibit high sensitivities. Compared to the direct ELISA, the step of binding secondary antibody to the primary antibody in indirect ELISA results in an increased sensitivity of the assay due to signal amplification. Thus, despite the complexity and time-consuming nature of indirect ELISA, it exhibits increased specificity and sensitivity making it more advantageous for *Orthoflavivirus* detection especially for differentiating between primary and secondary infections and detecting low antibody levels (Vista et al. 2023). Furthermore, sandwich ELISAs are considered to be the most sensitive. However, the selection of the format to be utilised is not just determined by sensitivity, but also by the intended purpose of detection. For example, the outcome may vary based on whether the goal is to detect antigens or antibodies, as well as the presence of capture and detector antibodies specific to the protein of interest. Additionally, ELISA is very useful tool in virus serology as it has the capability to identify antibodies of any isotype and in seroprevalence investigations (Fritzell et al. 2018). During the 2015–2016, ZIKV outbreak in the Americas, several studies emphasized the importance of using ELISA to distinguish and detect cross-reactivity between ZIKV and other co-circulating Arbovirus infections (Dengue and Chikungunya fever) (Waggoner et al. 2016; Oidtman et al. 2021). In the case of ZIKV, it is crucial to distinguish infection, especially in pregnant women, as ZIKV infection can result in congenital Zika disease, including microcephaly.

4.3.9 Status of Commercially Available Rapid ELISA

The MAC-ELISA test, developed by the Armed Forces Research Institute of Medical Sciences in 1989, is one of the earliest developed kits to detect IgM antibodies in cases of Dengue fever (Innis et al. 1989). The InBios International

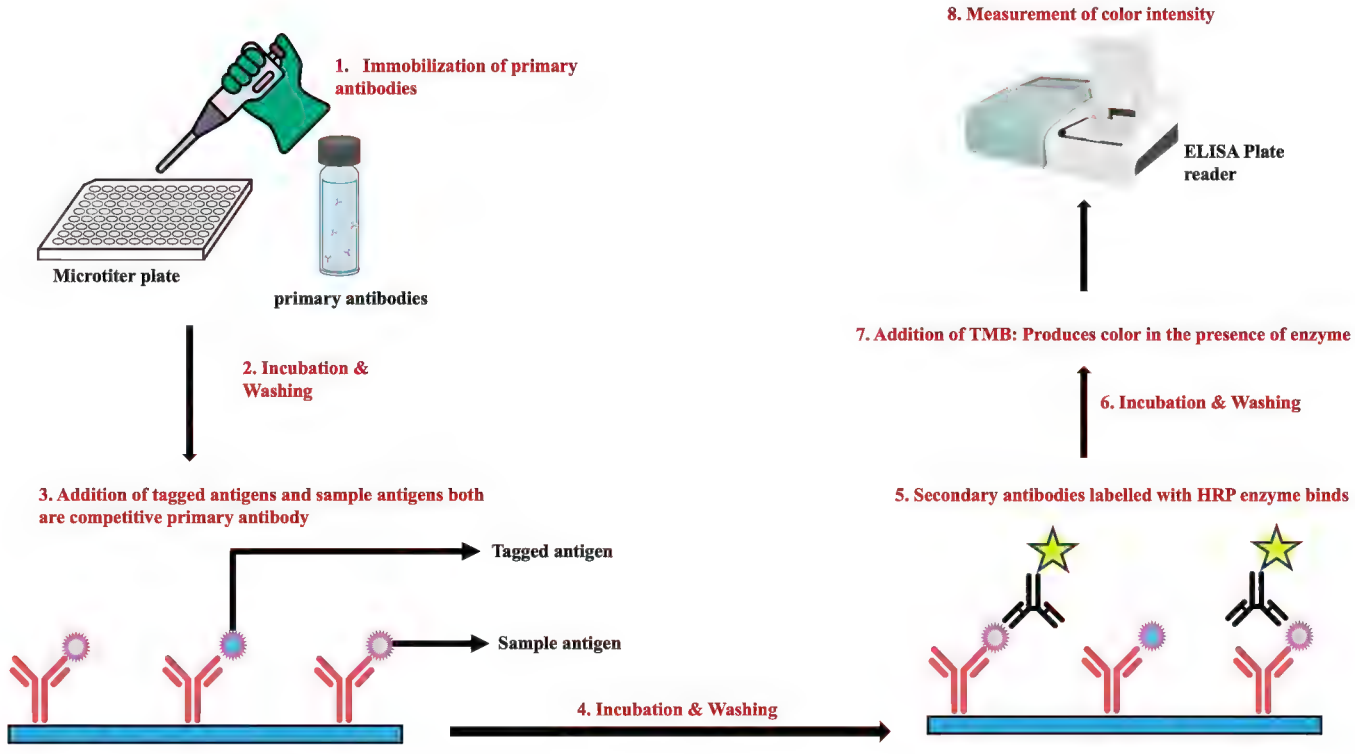
1. Immobilization of primary antibodies
Microtiter plate
primary antibodies
2. Incubation & Washing
3. Addition of tagged antigens and sample antigens both are competitive primary antibody
Tagged antigen
Sample antigen
4. Incubation & Washing
5. Secondary antibodies labelled with HRP enzyme binds
6. Incubation & Washing
7. Addition of TMB: Produces color in the presence of enzyme
8. Measurement of color intensity
ELISA Plate reader

Fig. 4.6 In sandwich ELISA, the plate is coated with primary antibodies followed by blocking addition of the antigen. Next, the *Orthoflavivirus*-specific primary antibodies are added followed by addition of enzyme bound secondary antibodies. These antibodies then bind with the primary antibodies at different region. The colour intensity is measured using ELISA plate reader

developed an US Food and Drug Administration (FDA)-approved assay known as DENV Detecta IgM Capture ELISA that detected the IgM antibody against DENV recombinant antigens, ensuring accurate and high-quality clinical results with a 96% sensitivity and 94% specificity (Namekar et al. 2013; Lee et al. 2019). Several IgG-based ELISA kits are available in the market for the detection of DENV, including kits from Standard Diagnostics, Panbio Inc., Inbios and Abcam, among others. The Standard Diagnostics (South Korea) kits have 81.2% sensitivity and 39.8% specificity for DENV. Also, Panbio Inc. (USA) introduced IgG-based ELISA kits with a sensitivity of 63.5% and a specificity of 95.3% (Blacksell et al. 2012). Lee et al. have performed a comparative study to assess the performance of several commercially available kits and found that the IgG tests exhibited many false-positive results and high cross-reactivity (Lee et al. 2019). Thus, IgG-based assays alone might not be able to determine the primary infection due to the potential cross-reactivity with other *Orthoflavivirus* IgG antibodies (Calvert et al. 2018).

A recent study conducted serosurveys to find the most used ELISA-based IgG kits for *Orthoflavivirus* diagnosis. The widely available PanBio Dengue IgG Indirect ELISA was found to be the most commonly used diagnostic method among commercially available kits for DENV, with 100% specificity and 97.9% sensitivity for secondary infections and 62% for endemic samples (Panbio Diagnostics). Nevertheless, the utilisation of this method in areas where the disease is prevalent may be limited due to its documented diagnostic efficacy. For detecting ZIKV, Euroimmun anti-ZIKV IgG ELISA was the predominant method with 100% sensitivity and 97% specificity confirmed by various studies (Low et al. 2021; Medialdea-Carrera et al. 2021). Apart from 0.7% cross-reactivity with DENV and 4% with JEV, no other instances of cross-reactivity were found with viruses, including WNV, YFV and tick-borne encephalitis virus (TBEV). Additionally, no instance of false positives was reported using this kit. The Focus Diagnostics and Euroimmun IgG ELISAs were found to be the most commonly used for WNV testing. The FDA tested the Focus Diagnostics kit using infected human samples or PRNT positive samples and demonstrated a sensitivity ranging from 36% to 100%. Nevertheless, the observed level of cross-reactivity was as high as 95% for DENV and 30%–57% for JEV, YFV and Saint Louis encephalitis virus (SLEV) (Vista et al. 2023). But the Euroimmun kit reported a sensitivity of 91% and specificity of 100% with low cross-reactivity with other *Orthoflavivirus* (Amin et al. 2020). In case of JEV, relatively few IgG ELISA kits are available and thus the serosurvey did not found any preferred kit for JEV diagnosis. This might be due to its lower occurrence rate primarily in Asia with some sporadic incidences in Australia. The limited number of JEV studies might be attributed to the fact that it was the first virus among the *Orthoflavivirus* to have a licensed vaccine and which led decrease in JEV cases (Campbell et al. 2011; Vannice et al. 2021). Additionally, the low levels of anti-JEV NS1 antibodies, lack of sufficient laboratory resources in rural regions where the disease is most widespread, further complicates the situation. In case of yellow fever, no such serosurveys have been done but few IgG ELISA kits are readily available in the market such as Euroimmun Anti-Yellow Fever Virus ELISA, Abcam IgG ELISA Kit.

4.3.10 Immunofluorescence Assay (IFA)

The immunofluorescence assay can detect viral antigens (direct IFA) or antiviral antibodies (indirect IFA) in the clinical samples (Reta et al. 2020). The direct IFA detects viral antigen in the sample and the process involved applying fluorescently labelled primary antibody to a fixed sample, such as cell or tissue culture. This step is followed by incubation and washing to eliminate any unattached antibodies. The target antigen is then observed directly using a fluorescent microscopy. This approach is simple and fast due to fewer steps and use of less reagents. However, its sensitivity may be compromised as the signal detection is exclusively dependent on the binding of the primary antibody. On the other hand, indirect IFA includes an extra step in which the primary antibody attaches to target antigen, and a secondary antibody linked with fluorescent dye, attaches to the primary antibody. The sensitivity of the assay is enhanced by this two-step approach as it allows for the binding of many secondary antibodies to a single primary antibody, hence enhancing the fluorescence signal (Fig. 4.7). However, indirect IFA is more intricate and time-consuming because of the additional incubation and washing steps. The most commonly employed fluorescent dye in the field of diagnostic virology is fluorescein isothiocyanate (FITC), which produces a vibrant yellow-green fluorescence. However, rhodamine, which creates a rich red fluorescence, is also an option. Following the application of a stain, the specimen is analysed using a fluorescence microscopy (Cella et al. 2013; Reta et al. 2020). The indirect IFA is currently more frequently utilised in scientific research than in clinical diagnostics. However, IFA is still not commonly utilised because of the inherent subjectivity involved in interpreting fluorescence signals, which rely on the quality and concentration of the antibody. Other limitations include the use of the right secondary antibodies, fluorochrome degradation, improperly purified antigens, using the assay on fixed cells, high expense, and the requirement of high expertise of laboratory personnel for handling specimens and interpreting outcomes (Odell and Cook 2013; Chan et al. 2022). Still, the quest for finding inexpensive and less tedious IFA combined with other techniques to identify antibodies against *Orthoflaviviruses* with increased specificity is still continuing.

In the case of WNV, both laboratory-developed and commercially available assays for IgM have demonstrated performance that is comparable to MAC ELISA (Koraka et al. 2002; Malan et al. 2003; Piantadosi and Kanjilal 2020). Most assays utilised are internally created by specific laboratories, employing WNV-infected cell cultures. Therefore, this technique lacks consistency when comparing data across different laboratories and would substantially benefit from an external quality assurance (EQA) evaluation, as demonstrated subsequently (Sambri et al. 2013). A commercially accessible IFA manufactured by Euroimmun in Germany was found to have a specificity of around 94% when compared to the plaque reduction neutralisation test (PRNT90). This comparison was made in a study conducted on 200 serum samples obtained during a human West Nile virus (WNV) outbreak in South Africa (Niedrig et al. 2007). A commercially available anti-ZIKV IIFT kit based on IFA assay has been developed by Euroimmun AG (Germany) with 96.8%

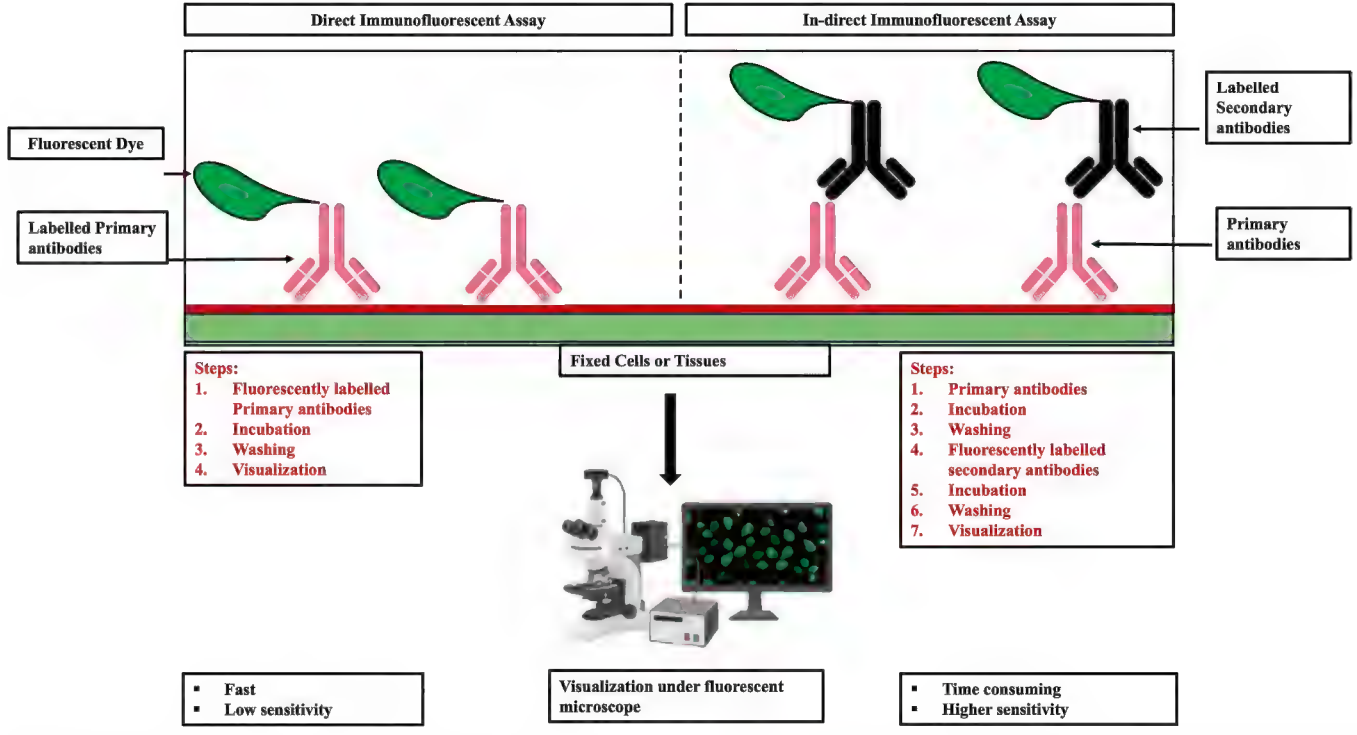

Fig. 4.7 In direct immunofluorescence assay (IFA), the florescent tagged primary antibodies are added into fixed cells or tissue samples, whereas in indirect IFA, the primary antibody is not tagged. The fluorescent tagged secondary antibodies are added after the addition of primary antibodies to increase sensitivity. After incubation and washing steps, the results are visualised under fluorescent microscope

sensitivity and 72.5% specificity for detecting the ZIKV. However, this assay uses the whole ZIKV as the antigen, which may or may not result in cross-reactivity with other *Orthoflavivirus,* as mentioned in the case of JEV IFA by Euroimmun (De Ory et al. 2018). In the case of JEV, a recent serological surveillance study was done in an endemic area using both commercially available Euroimmun IFA and ELISA (Kinsella et al. 2023). The Euroimmun IFA uses fluorescein-labelled anti-human IgM/G on BIOCHIP slides fixed with both non-infected and JEV-infected cells for detecting IgG/M against the JEV virus (Nakayama strain) (Euroimmun 2023). The results using IFA IgM and ELISA showed high sensitivity (100%) and minimum cross-reactivity (0–1.8%) with other endemic *Orthoflaviviruses* for acute JEV infection in the low endemicity study setting (Kinsella et al. 2023). Arai et al. conducted indirect IFA for the serological diagnosis of DENV in a population with a high frequency of arboviruses. They concluded that while indirect IFA was performed satisfactorily, ELISA by itself was sufficient for serological diagnosis when it came to clinical diagnosis of acute infection and detection of IgM antibodies. Indirect IFA might be a better option in place of ELISA, but it would also reduce IgM sensitivity and may lead to more false-negative IgM samples (Arai et al. 2019).

4.3.11 Hemagglutination Inhibition (HI) Assay

The hemagglutination inhibition (HI) assay exploits the ability of *Orthoflavivirus* to cause clumping of red blood cells (RBCs) and the subsequent prevention of this clumping by antibodies in the patient's blood. The assay involves several steps in which the serum samples from patients are collected and subjected to heat inactivation to eliminate complement activity. Next, these samples are diluted sequentially using phosphate-buffered saline (PBS). Each diluted serum sample is supplemented with a predetermined quantity of viral antigen followed by incubation to facilitate the binding of antibodies to the antigen. Next, a solution containing RBCs is introduced to each sample followed by incubation for 30–40 min (Mather et al. 2013; Sano and Ogawa 2014; Spackman and Sitaras 2020). If the serum contains particular antibodies against the *Orthoflavivirus*, these antibodies will attach to the viral antigens, thus inhibiting the virus from causing the RBCs to clump together. A positive result is shown by hemagglutination inhibition, which signifies the presence of antibodies against the specific *Orthoflavivirus* being tested. The agglutination is indicated by a smooth or jagged shield of cells or by an uneven button. Furthermore, the maximum dilution of serum sample which prevents hemagglutination, is considered the titer of the sample (Fig. 4.8). The HI assay is an easy, rapid and comparatively low-cost approach as it does not require specialised laboratory equipment or highly trained staff (Sano and Ogawa 2014; Spackman and Sitaras 2020). Although HI assay has been widely used for a long time, some studies have shown that it underestimates secondary infections when the cut-off recommended by the WHO was applied. This might be due to degradation of some HI antibodies below the detection threshold.

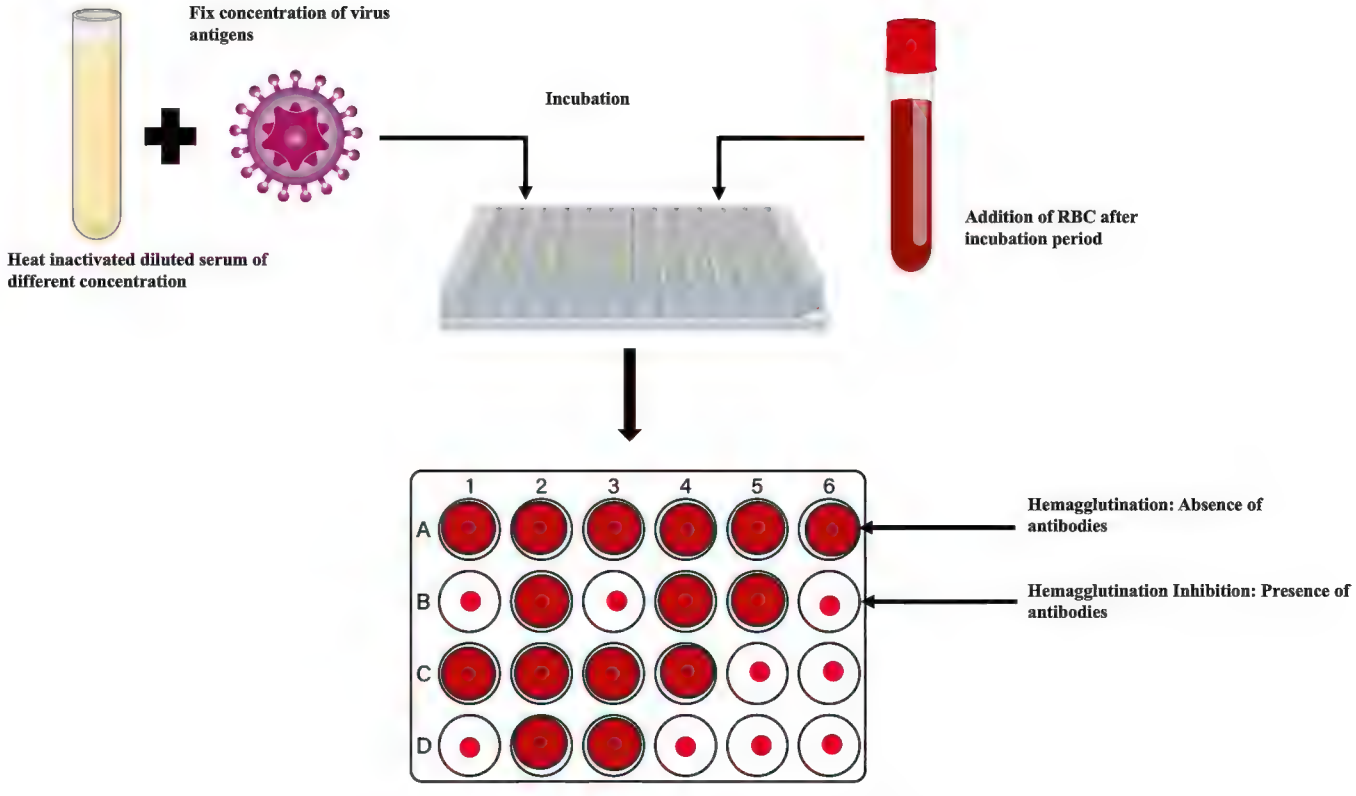

Fig. 4.8 In hemagglutination inhibition (HI) assay, the isolated serum is heated to eliminate the complement activity and then diluted sequentially in PBS. Next, a fixed quantity of antigen (virus) is added. Next, the RBC is introduced into the samples and incubated for 30–40 min. If the serum contains the antibodies, it will bind with the virus which inhibits the virus to cause RBC hemagglutination. RBC agglutination shows the absence of antibodies

Additionally, the HI antigens might not be readily available in some areas especially low-income countries (A-nuegoonpipat et al. 2006; Lukman et al. 2016). The authors Lukman et al. evaluated the precision of WHO HI criteria for acute and convalescent sera of DENV and compared it with WHO IgG ELISA and PRNT criteria. The HI assay and IgG ELISA demonstrated high accuracy (16 of 16) while identifying the primary DENV infection. However, the HI assay showed very low accuracy (23 of 73) compared to IgG ELISA (72 of 73) while identifying secondary infection. The author modified the cut-off criteria for both acute and convalescent HI sample results, which increased the accuracy for secondary infection from 31.5% to 93.2%. Still, the accuracy decreased from 100% to 81.3% for primary infections (Lukman et al. 2016).

4.4 Molecular Diagnosis

Researchers have developed several molecular amplification methods for detecting *Orthoflavivirus* in the past two decades. Molecular detection of *Orthoflavivirus* generally depends on the virus titre in the bloodstream. For instance, the titre of DENV in the bloodstream is comparatively higher than that of other viruses and remains longer in infected hosts. Extraction of nucleic acids is the first step in any molecular diagnostic or PCR experiment. The RNA from *Orthoflavivirus* can be isolated from the conventional phenol-chloroform method or various readily available viral RNA commercial kits, depending upon the preference of particular diagnostic laboratories (Sekaran and Artsob 2007; Daidoji et al. 2021). Generally, most commercial kits are based on the guanidinium organic extraction method and silica columns for RNA extraction and concentration. Extraction of viral RNA from samples prevents further exposure to potentially harmful live viruses.

In general, all variations of PCR consist of few basic steps including viral RNA extraction from infected sample, amplification using primers, reverse transcriptase, dNTPs, DNA polymerase and a fluorescent dye in case of quantification studies (Fig. 4.9). With the discovery of thermostable DNA enzyme, polymerase and advancement in PCR technique, the molecular detection of *Orthoflavivirus* has significantly improved (Mullis and Faloona 1987). A precise choice of primer sequences can be used to detect the specific virus and identify serotypes and polymorphisms. Rapidly detecting viral infection by PCR in the acute phase enables physicians to promptly and accurately initiate the treatment, which might significantly decrease severe disease. One of the most common examples is the Lanciotti primers designed for detecting and serotyping DENV (Lanciotti et al. 1992). Apart from use in general diagnostic and scientific research, PCR is often used for rapid identification and serotyping of virus during emergency. For instance, in 2003, in the United States, the PCR technique rapidly detected the presence of West Nile virus (WNV) in donated blood before it was used for transfusion. This helped to exclude the infected blood and prevent further infections or complications (Centers for Disease Control and Prevention (CDC) 2003).

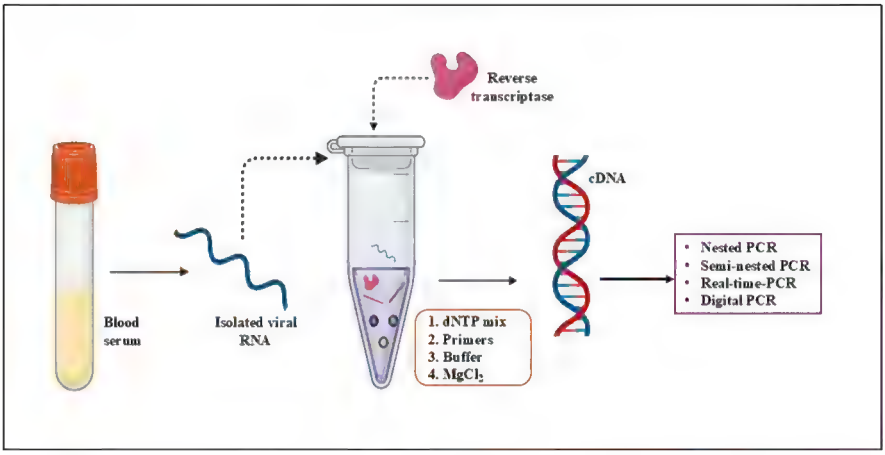

Fig. 4.9 The reverse transcriptase polymerase chain reaction (RT-PCR) utilises RNA molecule to produce multiple copies of cDNA. The isolated viral RNA from patient sample is added into the PCR mix which includes dNTPs mix, reverse transcriptase enzyme, primers, reagents in a single PCR tube and is amplified in the thermocycler. The resulting cDNA is further utilised for real-time PCR and nested PCR for viral detection

Although molecular methods are more convenient and comparatively less expensive than virus isolation and serological methods, they still have some cons. The incidence of sample mishandling and mix up increases while dealing with large number of samples leading false-positive results. Also, most of the molecular methods have multi-step and required high-cost PCR instruments. Additionally, the initial RNA extraction step may cause some interference in results and provide false-negative results due to insufficient sample (with extremely low titre), PCR inhibitors and presence of cellular constituents that could potentially compromise the amplification process. But these issues can be sometimes reduced by using commercially available RNA extraction kits, which also reduce the duration of the extraction process.

4.4.1 Reverse Transcriptase PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) is the most conventional method to detect RNA viruses from patient samples (Raengsakulrach et al. 2002). This method involves several steps after sample collection including RNA extraction, cDNA synthesis, external and nested PCR, followed by visualisation using agarose gel electrophoresis of amplified amplicons or Southern blot (Fig. 3.1). In nested PCR, the target region is amplified by using two sets of primers and thus double process of amplification (Fig. 4.10). A prime example of semi-nested PCR is detection of DENV using serotype specific consensus primers designed by Lanciotti et al. which

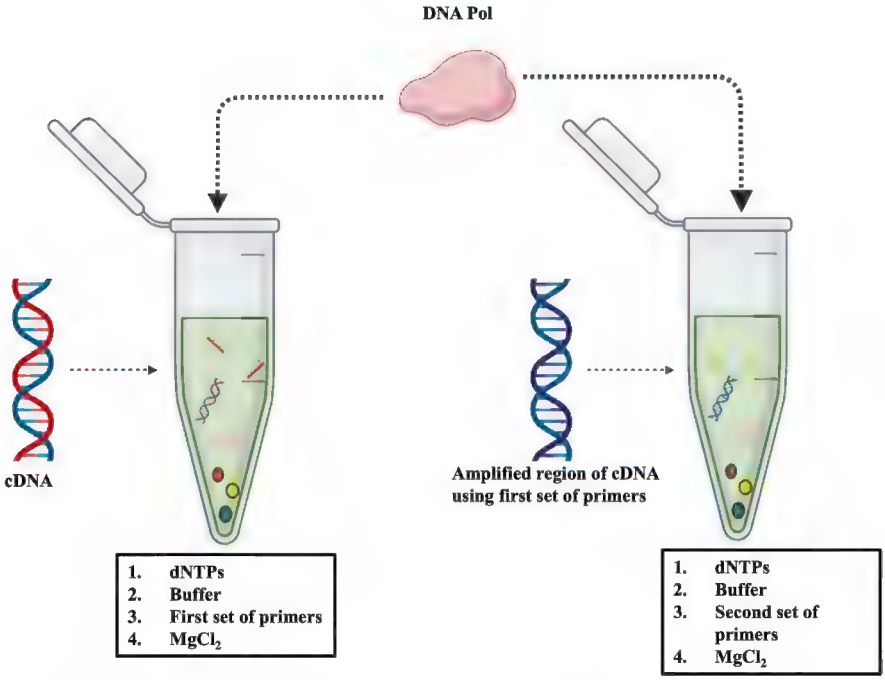

Fig. 4.10 Nested PCR involves two steps and utilises two different set of primers. After RNA isolation and cDNA synthesis, first round of PCR is performed. The resulting amplicon is again amplified using another set of primers. The final amplicons are visualised using agarose gel electrophoresis for viral detection and serotyping

targets the conserved region of CprM gene and amplify a 511 bp in first step and 119–485 bp of DENV 1–4 in second step of PCR. These primers are widely used in laboratories for diagnosis and research for detecting all the serotype and has 93–100% sensitivity among DENV 1–4 (Lanciotti et al. 1992). The use of conventional two-step PCR is currently declining due to its time-consuming nature, multiple steps and susceptibility to contamination resulting from the sequential handling of amplicons. Even though the two-step PCR provided specific results by reducing non-specific or improper primer binding, the issue of carry-over amplicons from the previous step might also result in PCR errors or false positives (Mori et al. 2021). A study by Harris et al. reported false-negative results of DENV infection using the two-step PCR and designed one-step PCR targeting CprM region of DENV which improved the specificity, allowed handling of large number of samples simultaneously and minimised false-positive results (Harris et al. 1998). Since then, several primers were designed for one- or two-step rapid detection of *Orthoflavivirus* by PCR (Lanciotti et al. 2000; Johnson et al. 2001; Sekaran and Artsob 2007; Faye et al. 2008; Nunes et al. 2011; Waggoner et al. 2013; Méndez et al. 2013; Sousa et al. 2020; Mori et al. 2021).

Multiplex PCR can differentiate virus and its serotypes by simultaneously amplifying and detecting numerous target amplicons. It is very convenient for areas where there is co-circulation of multiple serotypes and other arthropod borne viruses are common. Genus-specific universal primers may amplify sequences from a wide range of *Orthoflaviviruses* from diverse genetic subgroups. For instance, Daidoji et al. designed a pan-*Orthoflavivirus* (i.e., DENV, ZIKV, JEV, WNV, YFV and TBEV, among others) primers against the NS5 gene, which was able to detect the viral genome in live *Orthoflavivirus* infected cells and from crude mosquito homogenates (Daidoji et al. 2021). The primers also produced better, and brighter PCR bands compared to other well-known primer pairs. Furthermore, the authors Scaramozzino et al. designed a pan-*Orthoflavivirus* (including DENV, ZIKV, WNV, Yellow fever and JEV, among other *Orthoflaviviruses*) hemi-nested PCR primers that specifically targets the conserved region of NS5 gene, demonstrating high sensitivity (Scaramozzino et al. 2001). Further, Calvo et al. designed primers for faster and accurate nested-PCR method for simultaneously identifying DENV, ZIKV and CHIKV in a single sample using clinical samples. In the first step of the multiplex RT-PCR, first step amplified CprM, E and E1 genes by using set of six outer primers. This was followed by second round of amplification using inner primers for each virus and was able to detect both single, co-infections and triple-infection simultaneously. Thus, this method is comparatively cost-effective and rapid and have potential to be a valuable method for identifying *Arbovirus* infections in areas where these co-circulate. Several other studies reported new primers and RT-PCR methods with modified detection methods (such as microwell hybridisation assay) for simultaneous detection of serotypes, *Orthoflavivirus* or *Arbovirus* infections and co-infections (Saxena et al. 2008; Nunes et al. 2011; Nunes et al. 2018; Islam et al. 2020; Calderon-Ruiz et al. 2023).

4.4.2 *Quantitative Reverse Transcription PCR (RT-qPCR)*

Quantitative reverse transcription PCR (RT-qPCR) is the most widely used diagnostic method due to its qualitative and quantitative nature of results. It is more robust and sensitive than conventional endpoint PCR, with multiplexing ability, automation and lower contamination risk. It is more suitable for large sample testing (Sekaran and Artsob 2007; Gyawali and Taylor-Robinson 2017; Mori et al. 2021; Kabir et al. 2021). In general, similar to conventional PCR, the RT-qPCR steps include RNA extraction, cDNA synthesis, followed by target amplification using primers and quantification using specialised qPCR instrument. RT-qPCR is the relative quantification method that measures the gene expression difference between two samples by calculating the fold change (Fig. 4.11). The threshold cycle number [Ct] is crucial for assessing the qualitative outcome and determining the quantity of RNA targets in the sample. Therefore, it is possible to determine the quantity of viral RNA or viral load in the test sample. Moreover, qPCR also eliminates post-PCR procedures like electrophoresis or blotting for the final detection of amplicons

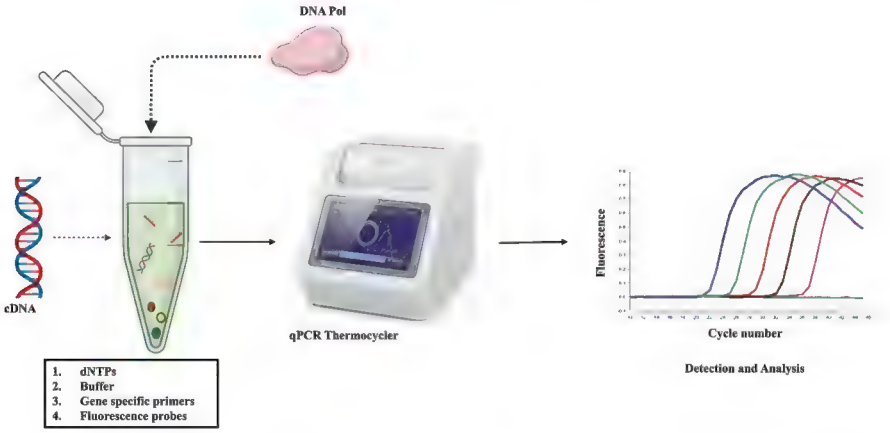

Fig. 4.11 Quantitative or real time PCR (RT-qPCR) is used for both viral detection and quantification. The PCR mixture contains cDNA, primers, dNTPs, DNA polymerase and fluorescence probe in a single PCR tube. The cDNA is amplified using a thermocycler using the PCR standard protocol. The amplification plot so obtained is used for relative quantification of virus

(Lanciotti 2003; Shukla et al. 2016; Gyawali and Taylor-Robinson 2017). Two main types of real-time methods are the TaqMan probe and the SYBR green assay. The SYBR green method is comparatively easy and cost-effective compared to other types of qPCR. It allows for the simultaneous amplification of numerous targets, which may be identified by analysing the melting temperature (Tm) after amplification. The conventional end-point PCR can also be modified into RT-qPCR, such as the pan-*Orthoflavivirus* PCR developed by Scaramozzino et al. (2001). A more recent detection method involves using fluorescent probes that can be detected in real-time. The TaqMan probe method uses a fluorogenic probe that binds at a specific site, thereby improving the sensitivity and specificity of assay. TaqMan assays have been successfully developed to identify many *Orthoflavivirus*, including DENV, YFV and JEV (Houng et al. 2001; Huang et al. 2004; Kong et al. 2006; Mori et al. 2021).

The monoplex RT-qPCR (CDC reference assay) developed by Lanciotti et al. is the most widely used diagnostic method for detecting ZIKV (Lanciotti et al. 2008). Others also developed qPCR-based methods for detecting ZIKV from mosquitoes or clinical samples targeting NS1, NS5 and E gene (Faye et al. 2013; Pyke et al. 2014; Tappe et al. 2014; Waggoner and Pinsky 2016; Corman et al. 2016; de Moraes et al. 2018). A study compared and reported NS1-based test developed by Pyke et al. exhibits the highest sensitivity in detecting the circulating lineage of the virus (de Moraes et al. 2018). Vazquez et al. conducted a study to detect all known WNV lineages by designing primers against the genome's 3′ untranslated region (3'UTR). They validated it using plasma, serum, human cerebrospinal fluid and biopsies with sensitivity ranging between 1.5 and 15 copies (depending on specific WNV lineage) per reaction (Vázquez et al. 2016). In case of DENV diagnosis, several monoplex RT-qPCRs (both pan-DENV and serotype identifier) has been published with sensitivity up to 90% (Shu and Huang 2004).

It has been reported that multiplex qPCR outperforms conventional RT-PCR in clinical and analytical performance and workflow. Fully automatic RT-qPCR techniques for rapid identification of DENV, ZIKV, WNV, JEV and YFV in acute-phase serum samples have also been documented (Houng et al. 2001; Drosten et al. 2002; Wang et al. 2002; Shu et al. 2003; Chao et al. 2007; Amin et al. 2020; Xu et al. 2022). As per conventional PCR, qPCR primer sequences can also be specified to detect DENV to a threshold of <100 plaque-forming units (Yong et al. 2007; Gyawali and Taylor-Robinson 2017). Furthermore, Johnson et al. designed a quantitative four-plex real-time PCR method to detect DENV infection from serum samples with the same sensitivity as monoplex reactions. The CDC modified this method (approved by the FDA) for up to 90% sensitivity to detect all globally circulating variants of DENV1–4 (Johnson et al. 2005; Santiago et al. 2013).

Nevertheless, there are still some drawbacks in RT-qPCR. In the SYBR green method, the primers can attach to any double-stranded DNA, such as primer dimers, potentially leading to overestimating the DNA concentration. Even though extensive database searches design SYBR green and conventional PCR primers, the clinical samples will contain other non-targeted RNA, including human rRNA, which might affect primer specificity. The TaqMan probe method also has many disadvantages, including its expensive nature, dependence on fluorogenic probes and the requirement for specialised equipment. Furthermore, the capacity to develop a multiplex TaqMan test is limited to incorporating a range of one to four unique targets per assay, contingent upon the equipment employed (Sekaran and Artsob 2007).

4.4.3 Digital PCR (dPCR)

Digital PCR is a new and emerging endpoint qPCR method based on the absolute quantification of target viral genes. It measures absolute gene expression levels in terms of the exact copy number (Sykes et al. 1992; Vogelstein and Kinzler 1999; Sedlak and Jerome 2013; Vynck et al. 2016; Quan et al. 2018). In dPCR, the sample is initially divided into independent separate PCR sub-reactions where each sub-reaction contains few to no target sequences. Each sub-reaction gives positive or negative signal. Following PCR, the sub-section showing amplification is used to measure the concentration of the target sequence or simply number of DNA molecules in original sample is directly calculated from the number of negative and positive reactions using Poisson's statistics (Vynck et al. 2016; Quan et al. 2018). Sample partitioning effectively concentrates the target sequences within the separated microreactors (Fig. 4.12). This concentration effect minimises template competition, allowing for the identification of uncommon mutations within a pool of wild type sequences (Sykes et al. 1992; Vogelstein and Kinzler 1999; Quan et al. 2018). Life Technologies (Carlsbad, California) and Fluidigm Corporation (San Francisco, California) developed microfluidic-based dPCR system. These instruments use advanced chips with microfluidics channels and divide samples into individual reaction wells, delivering very small amounts of sample (in nanolitres)

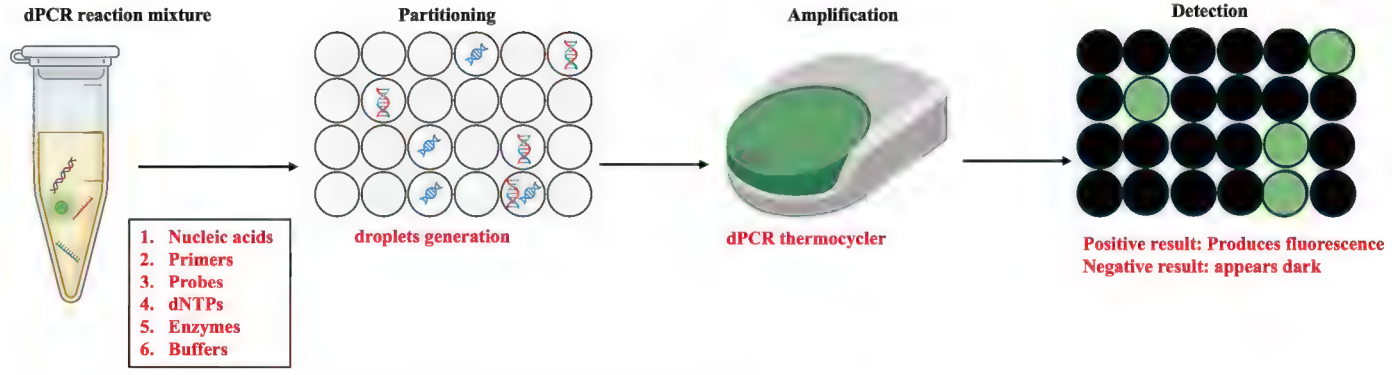

Fig. 4.12 In digital PCR (dPCR), the absolute detection of target DNA can be done. The reaction mixture contains templates of nucleic acids, primers, probes, dNTPs, enzymes and buffer. This reaction mixture is then partitioned into micro-droplets. Each droplet works like a micro-reaction for amplification. The amplified target DNA produces fluorescence while negative reactions appear dark

(Sedlak and Jerome 2013). The authors Hui et al. designed and compared dPCR and qPCR targeting the NS5 gene of ZIKV. The result showed that dPCR had significantly high sensitivity in detecting low concentrations of ZIKV, but RT-qPCR showed a strong linear relation only at concentration above 10 copy/μl (Hui et al. 2018).

Recently, the availability of commercial dPCR has increased its use in the clinical diagnostic applications because it can detect very low viral load and few mutants in the sample, unlike qPCR. Unlike qPCR, where the quantification is influenced by the instrument used, fluorescent reporter dye and efficiency of the assay; dPCR simply involves counting the number of amplification reactions or fluorescent droplets. Thus, knowing the total number of droplets and volume is all that is needed for dPCR. Compared to relative qPCR, it is highly sensitive, reproducible, consistent, accurate, less time consuming, less labour intensive and can detect low titre positive samples also. It does not require standard curve analysis and has a high tolerance to PCR inhibitors. Moreover, it may also provide a greater resistance to inhibitors found in a sample (Sedlak and Jerome 2013; Vynck et al. 2016; Quan et al. 2018; Mori et al. 2021). However, the technology is still new and is comparatively much expensive than conventional PCR or qPCR. Also, the cost per sample of RT-dPCR might be up to 10 times more than that of conventional qPCR (Abachin et al. 2018).

4.5 Isothermal Amplification (IA) Techniques

The isothermal amplification (IA) techniques are one of the emerging fields in *Orthoflavivirus* detection and diagnosis due to their sensitivity, cost-effectiveness, easy procedure and ease of detection. Contrary to PCR-based procedures, IA methods utilise a heating block set at a constant temperature, eliminating the requirement for high-precision instruments like thermocyclers. However, these techniques are relatively new and needs further advancement and optimisation for diagnosis, low cross-reactivities and false-positive results.

4.5.1 Nucleic Acid Sequence-Based Amplification (NASBA)

One emerging IA technique is the nucleic acid sequence-based amplification (NASBA) method. NASBA is a transcription-based amplification (TBA) method that imitates retroviral RNA replication and is designed to specifically target RNA sequence, i.e., it can directly amplify RNA. Thus, a major advantage of NASBA is that it has single step only and eliminates the need for costly thermal cycler. This thermally stable RNA amplification procedure involves an initial denaturation stage at 65 °C for 5 minutes and elongation at 41 °C for 90 minutes. It amplifies the DNA using three distinct enzymes: reverse transcriptase, RNase-H and T7-RNA polymerase. Combining these enzymes with the RNA leads to up to 12-fold increase

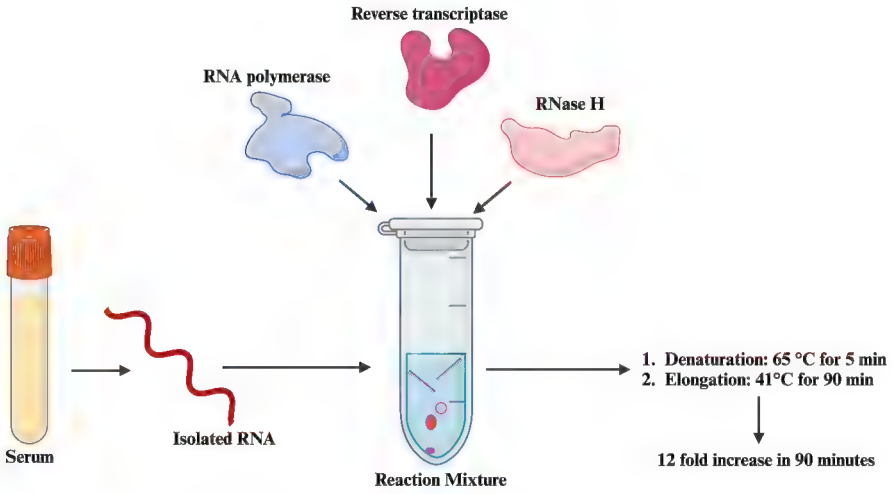

Fig. 4.13 In nucleic acid sequence-based amplification (NASBA), the isolated RNA from patient serum is added to a reaction mixture containing three enzymes viz. RNA polymerase, reverse transcriptase and RNase H with other reagents which amplifies viral RNA. The reaction is carried out in a hot water bath at 65 °C for 5 min for denaturation step followed by elongation step at 41 °C for 90 min. The result may yield 12-fold increase in viral particles can be detected by real-time fluorescent-labelled probes, electrochemiluminescence (ECL) signal count or agarose gel electrophoresis

within 90 min. The result thus can be detected by real-time fluorescent-labelled probes, electrochemiluminescence (ECL) signal count and agarose gel electrophoresis (Fig. 4.13) (Compton 1991; Varghese et al. 2023).

Efforts are going on to optimise the NASBA assay to detect *Orthoflavivirus* efficiently. The authors Wu et al. developed and improved the semiquantitative NASBA method to amplify DENV 1–4 by using a set of universal primers and distinguishing them based on serotype-specific capture probes. The diagnostic sensitivity and specificity for DENV detection were 98.5% and 100%, respectively, from serum/plasma samples (Wu et al. 2001). Further, Baeumner et al. developed a method using a lateral flow biosensor assay to rapidly detect DENV 1–4 from blood samples within 15 min (Baeumner et al. 2002). Another DENV NASBA assay demonstrated a 1 PFU/ml sensitivity for all four DENV serotypes. However, this assay was only evaluated on infected tissue culture cells (Jittmittraphap et al. 2006). Very few yet successful studies have been done regarding the application of NASBA ZIKV detection (Pardee et al. 2016). However, NASBA also has some drawbacks as the primary concern is viral RNA integrity and controlling temperature of the reaction. Thus, it cannot be considered as the truly IA techniques due to the denaturation step and thermolabile nature of the enzymes used for amplification. Also, NASBA is only effective for amplifying short oligonucleotides (120–250 bp) (Dunbar and Das 2019; De Felice et al. 2022).

4.5.2 *Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)*

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is also based on isothermal amplification and does not require a thermal cycler. It can amplify RNA and DNA and is a modified version of the nucleic acid amplification technique. The technique involves a set of four or six primers, consisting of outer and inner primers, to increase the amplification by directing at several target sequences and subsequently improving specificity (Notomi et al. 2015). The assay relies on the uninterrupted synthesis and utilises strand displacement of replicated DNA without the requirement to separate the strands using heat and amplifies the stem-loop structure of the target sequence at a consistent incubation temperature of 60–65 °C, which typically lasts for an hour (Fig. 4.14). The reagents and enzymes (e.g., Bst polymerase) used in LAMP are more resistant to inhibitors commonly found in biological samples than Taq polymerase (Notomi et al. 2015; Teoh et al. 2015). During amplification, magnesium pyrophosphate is generated as a secondary product or byproduct, leading to the cloudiness or turbidity in the sample. Therefore, the measurement of the amplified product is determined by the degree of turbidity, which can be detected using a turbidimeter (Mori et al. 2001, 2021; Varghese et al. 2023). Besides detecting turbidity, colorimetric detection can also be done using hydroxy napthol blue, which causes a transition in colour from violet to sky blue (Goto et al. 2009).

Parida et al. successfully developed a quantitative assay and applied a single-step, four-tube RT-LAMP method using four DENV serotype-specific primers (Parida et al. 2005). Further, Teoh et al. developed a qualitative assay single-step RT-LAMP method using a single-tube set of nine primers to identify all four serotypes of DENV, including their distinct genotypes. They targeted the 3′ UTR region

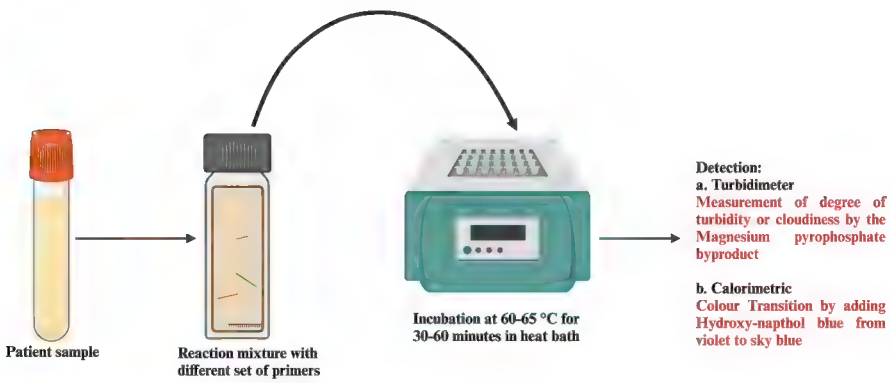
Patient sample
Reaction mixture with different set of primers
Incubation at 60-65 °C for 30-60 minutes in heat bath
Detection:
a. Turbidimeter
Measurement of degree of turbidity or cloudiness by the Magnesium pyrophosphate byproduct
b. Calorimetric
Colour Transition by adding Hydroxy-napthol blue from violet to sky blue

Fig. 4.14 In reverse transcription loop-mediated isothermal amplification (RT-LAMP), the sample containing viral RNA is directly added to the reaction mixture. The reaction mixture contains different sets of primers, enzymes and other reagents for amplification. This reaction mixture is then allowed to incubate in hot water bath for 30–60 min at 60–65 °C. The final viral detection is done by turbidimeter or calorimetric method

of DENV and showed significant results (Teoh et al. 2015). Song et al. created an impressive RT-LAMP assay focusing on a consistent area on the E gene. This assay may be performed on a microfluidic Point-of-care (POC) diagnostic system. When tested with different concentrations of ZIKV in saliva, the POC diagnostic system had a sensitivity of less than 5 PFU per sample. Another method utilised was a streptavidin-magnetic nanoparticle-based LAMP-AC susceptometry assay (Song et al. 2016). Lee et al. described another pioneering system which integrated RT-LAMP with lateral flow immunoassay to precisely detect ZIKV at a sensitivity of less than 1 copy within 35 min (Lee et al. 2016). Estrela et al. have presented a method that reduces time, yielding findings in just 10 min without RNA extraction. Several RT-LAMP techniques were developed to differentiate between the African and Asian lineages of the ZIKV by targeting conserved sequences, RT-LAMP-based SNPs typing technique (Chotiwan et al. 2017; Estrela et al. 2019; Bui et al. 2020). Additionally, a study designed an RT-LAMP tool that can detect and distinguish JEV, WNV and all four serotypes of DENV simultaneously (Li et al. 2011). Others have also similar RT-LAMP assay for YFV, WNV and JEV (Kwallah et al. 2013; Mori et al. 2021).

In addition, the RT-LAMP method has been extensively modified to utilise it as smartphone-based detection system for achieving prompt results. In the study by Ganguly et al., a microfluidic device was used to perform detection of ZIKV, DENV-1, DENV-3 and CHIKV directly from whole blood samples with minimal sample processing on a diagnostics card followed by RT-LAMP on the same card which contains pre-dried primers specific to viral targets. Next, a smartphone was used to capture the real-time photos of amplification and presents a visual representation of assay. The result showed high level of specificity and clinically significant sensitivity and was able to detect 1.56e5 PFU/ml for ZIKV (Ganguli et al. 2017). Similarly, in another study a microfluidic device made of wax-printed paper was used for RT-LAMP assay to detect ZIKV. The assay used diluted (10%) human blood plasma, tap water and human urine, and optimised the characteristics of different paper microfluidic chips, including type of paper used, pore size and dimensions of channels to ensure effective filtering of biological materials as the flow is driven by capillary action. After ZIKV has moved and arrived at the detection area of the paper microfluidic chip, it was cut out to allow the addition of an RT-LAMP mixture including a pH indicator. The chip was then placed on a hot plate set at a temperature of 68 °C. The successful amplification process resulted in observable colour changes within a time frame of 15 min, which were then measured using smartphone imaging. The detection limit was as low as 1 copy per μL. Similar results were shown by other studies based on smartphone detection for detection of *Orthoflavivirus* (Priye et al. 2017; Kaarj et al. 2018).

Additionally, it may be used for point-of-care patient surveillance to determine the presence of *Orthoflavivirus* or specific biomarkers in whole blood. Thus, this RT-LAMP assay is comparatively more direct, faster, efficient and cost-effective, and thus allows rapid and precise amplification of the viral genome which can be further optimised for *Orthoflavivirus* detection. Furthermore, RT-LAMP does not require any specialised instrument, thereby making it suitable alternative of PCR or ELISA for prompt detection.

4.5.3 *RT-Recombinase Polymerase Amplification (RT-RPA)*

The recombinase polymerase amplification (RPA) assay has recently emerged as a new and innovative IA approach for detecting viral infections, including *Orthoflaviviruses*. For the detection of RNA viruses reverse transcription step is required, and the assay is known as reverse transcription recombinase polymerase amplification (RT-RPA). In general, after patient sample collection, the viral RNA is extracted followed by cDNA synthesis and amplification using RPA enzymes in a single reaction mixture. The RPA assay requires a steady temperature between 37 °C and 42 °C for exponential amplification and uses a set of primers with three enzymes responsible for DNA synthesis, recombination and repair. The enzymes used are single-stranded binding protein (SSB), recombinase and strand-displacing polymerase (Fig. 4.15). The final result can be detected using agarose gel electrophoresis (Mori et al. 2001; Piepenburg et al. 2006).

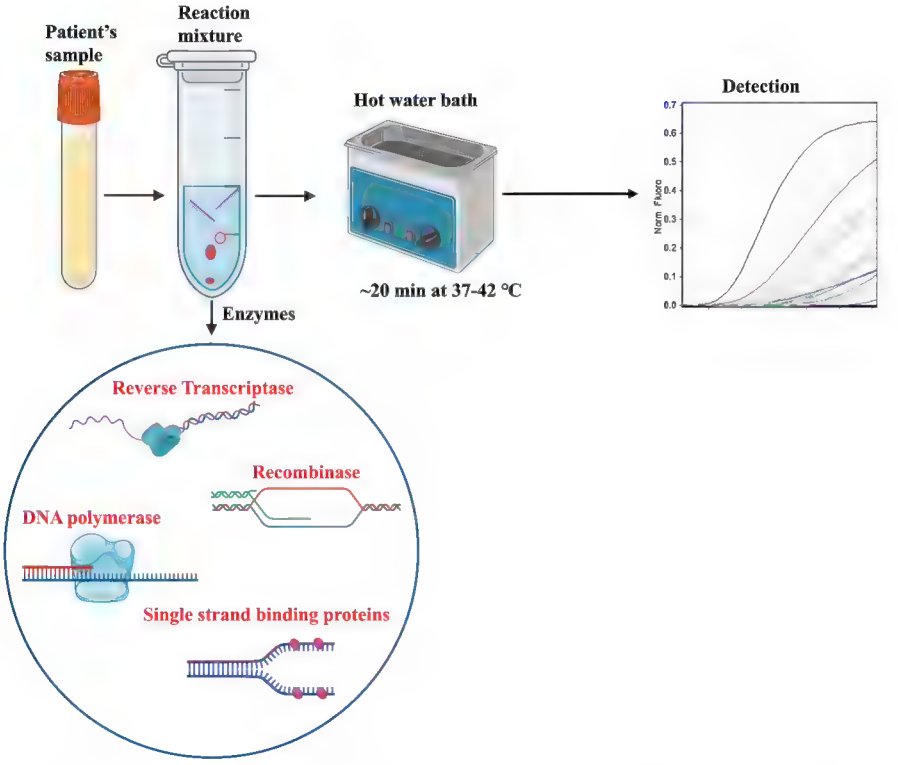

Fig. 4.15 In reverse transcription recombinase polymerase amplification (RT-RPA), a single reaction mixture contains all the necessary enzymes, primers and other reagents for amplification of viral RNA. The enzymes used are reverse transcriptase, recombinase and DNA polymerase including others. The reaction mixture is incubated in a hot water bath for about 20 minutes at 37–42 °C. The final result can be detected using agarose gel electrophoresis

In 2017, Abd El Wahed et al. developed a rapid RT-RPA assay for the detection of ZIKV using urine samples, which yielded results within 15 min. The assay showed high specificity and sensitivity of 100% and 92%, respectively, and detected as few as 21 copies of ZIKV. Also, no cross-reactivity with other *Orthoflavivirus*, alphaviruses and other *Arboviruses* were observed with 100% specificity for ZIKV (Abd El Wahed et al. 2017). Teoh et al. developed an RT-RPA assay for detecting DENV and evaluated serum samples from symptomatic patients. The assay rapidly identified as few as 10 copies of DENV RNA within 20 min. The designed primers and exo-probe were able to identify the currently circulating 12 genotypes of DENV. Additionally, no false-positive results were obtained when these primers and probes were tested against other *Orthoflaviviruses* (Teoh et al. 2015). In another study with 110 WNV sample, the RPA reaction was successfully conducted at a temperature of 39 °C for a duration of 15 min using a real-time thermal cycler. The assay sensitivity was high and was capable of detecting as low as 10 copies. The concordance between RT-RPA and the reference RT-qPCR assay was demonstrated to be exceptionally high with RPA being 10–20 times quicker than RT-qPCR (Tomar et al. 2021).

The RT-RPA assay is still in its early stages of development, yet it offers several advantages over the RT-LAMP assay. RPA assay can be performed within 20 min compared to RT-RPA, which requires at least 60 min (Piepenburg et al. 2006; Teoh et al. 2015). It is also easier to perform. For instance, the ready-to-use RT-RPA reaction mixture simplifies the assay workload and reduces pipetting errors. The RT-RPA assay offers several benefits that make it an appealing diagnostic test for the routine detection of DENV RNA, particularly in healthcare settings with limited resources. It can be easily conducted by personnel with less expertise. Furthermore, the RT-RPA assay is more suitable for downsizing point-of-care applications than the RT-LAMP assay, as it necessitates less energy input.

4.6 Conclusions

The robust advancement of diagnostic assays in molecular virology is necessary as it greatly aids in the fight against the viral infections. The classic virus isolation method is still a challenging and time-consuming procedure that necessitates appropriate biocontainment facilities. Molecular approaches such as PCR, RT-qPCR and digital PCR that rely on the detection of viral RNA are still considered as the most effective tools for quickly identifying the *Orthoflavivirus* infections and provides a balance between speed, sensitivity and specificity. However, the use of PCR techniques is constrained by the necessity for high-cost specialised equipment, skilled staffed, need for costly reagents and multiple steps for detecting the amplified products. These obstacles have limited its use, particularly in distant regions and low-income countries. Further, the IgG, IgM and NS1 dependent serological assays are typically used for rapid detection and high sensitivity but are not suitable for making a differential diagnosis as they have the potential to produce cross-reactivity. Furthermore, currently IA technologies are considered comparatively more

cost-effective, efficient and suitable due to its less tedious protocol and minimum pre-treatment step of RNA extraction and purification. However, these IA techniques are more likely to contain amplification inhibitors which may result in false-negative results. Thus, all these diagnostic methods should be further optimised and enhanced for accurate and differential diagnosis of *Orthoflavivirus* infections. Also, from our perspective the RT-LAMP assay is the most rapid, cost effective, sensitive, less tedious detection method with low false-positive rates and should be further improved for *Orthoflavivirus* diagnosis.

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Conflict of Interest None to declare.

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Chapter 5
Molecular Diagnosis of Human Immunodeficiency Virus (HIV) Infection

Panchami P. Bhat and Subhash Mehto

Abstract In recent decades, the field of molecular diagnostics for human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) has seen significant progress, revolutionizing the detection and management of HIV infection. Techniques like polymerase chain reaction (PCR) and nucleic acid amplification tests (NAATs), have greatly improved the accuracy and sensitivity of HIV detection. These techniques can identify the presence of the virus even during the window period, which is the early phase of infection when antibodies have not yet developed and traditional antibody tests might fail to detect the virus. Molecular assays are essential for monitoring viral load, detecting drug resistance, mutations, guiding treatment decisions, and improving patient care in HIV management. This chapter underscores the pivotal role of molecular diagnostics in the global efforts to combat HIV/AIDS. By enabling timely and precise diagnosis, facilitating effective treatment strategies, and improving patient outcomes, these techniques are vital in addressing the HIV epidemic. The advancements in molecular diagnostics not only enhance the quality of life for those living with HIV but also contribute to broader public health goals, such as reducing transmission rates and moving closer to an AIDS-free generation.

Keywords Human Immunodeficiency Virus · Acquired Immunodeficiency Syndrome · Polymerase Chain Reaction · ELISA

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5.1 Introduction

Human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) remains a major global health challenge, impacting millions of lives worldwide. Since the emergence of the epidemic in the early 1980s. It has claimed over 40 million lives and currently affects approximately 39 million people as of 2022. The virus disproportionately affects marginalized and vulnerable populations, particularly in sub-Saharan Africa, which bears the highest burden of the disease. Apart from severe health consequences, HIV/AIDS also has significant social and economic impacts. It exacerbates poverty, reduces labor productivity, and strains healthcare systems, especially in resource-limited settings (RLS). The epidemic also fuels stigma and discrimination, hindering individuals' access to care and quality of life. Despite these challenges, significant progress has been made through global initiatives aimed at prevention, treatment, and education. Antiretroviral therapy (ART) has turned HIV into a manageable chronic condition for many, enhancing life expectancy and quality of life. Ongoing efforts to combat HIV/AIDS emphasize the importance of equitable access to healthcare, continued research, and the implementation of comprehensive public health strategies to ultimately end the epidemic. HIV is a progressive disease that can lead to AIDS if left untreated. Timely diagnosis and treatment can slow down or even halt the progression of the disease, preventing the development of AIDS-related complications such as opportunistic infections. Individuals who are unaware of their HIV status are more likely to engage in behaviors that can transmit the virus to others. Timely diagnosis allows individuals to take measures to prevent transmission, such as using condoms and practicing safer sex, and consider interventions like pre-exposure prophylaxis (PrEP) for their partners. Moreover, early identification of HIV infection allows for immediate initiation of antiviral therapy (ART), which helps suppress the virus, lower viral load, and restore the immune system. Starting treatment early can significantly improve long-term health outcomes and reduce the risk of virus transmission to others. Additionally, early diagnosis and treatment can lead to cost savings by preventing costly hospitalizations and complications associated with advanced disease, as well as reducing the need for expensive interventions to manage HIV-related complications.

5.2 HIV Biology and Pathogenesis

In 1983, a significant breakthrough in virology occurred with the isolation of HIV-1, a monumental achievement in the quest to understand and combat infectious diseases (Barré-Sinoussi et al. 1983; Gallo et al. 1983). Following this landmark discovery, the strong association between HIV-1 and AIDS was established in 1984, reshaping the landscape of medical research and public health interventions (Gallo et al. 1984; Montagnier et al. 1984). Subsequently, in 1986, another significant

milestone unfolded with the identification of a related virus known as HIV-2, originating and confined to West Africa such as Portugal (Clavel et al. 1986). This discovery broadened our understanding of the virological diversity underlying immunodeficiency syndrome, highlighting the complexity of viral pathogens and their regional variations. Both HIV-1 and HIV-2, although distinct entities, are unified within the taxonomic classification of the Retroviridae family and subfamily Orthoretrovirinae, emphasizing their evolutionary relationship and shared biological characteristics. Retroviruses represent a distinct group of viruses characterized by their enveloped structure and utilization of RNA as their genetic material. Retroviruses are further classified into various genera based on their biological properties and genetic characteristics. HIV-1 and HIV-2 belong to the Lentivirus genus, named after the Latin word "lentus" meaning "slow" reflecting the delayed onset of disease symptoms characteristic of lentivirus infections. What sets them apart is their unique replication process, which involves the conversion of RNA into DNA, a step facilitated by the enzyme reverse transcriptase.

5.3 The Structure and Genome of HIV

HIV is a complex retrovirus belonging to the lentivirus family, known for its slow progression and chronic infection. Structurally, HIV has an outer lipid envelope composed of viral glycoproteins gp120 and gp41, which are crucial for virus attachment and entry into host cells. Inside the envelope, matrix protein encases the viral core. The core contains two single-stranded RNA molecules, the virus's genetic material, along with essential enzymes such as reverse transcriptase, integrase, and protease (Fig. 5.1). HIV's genome is approximately 10 kilobase (kb) positive sense viral RNA which contains nine genes: three major structural genes (gag, pol, and env) and six regulatory/accessory genes (tat, rev, nef, vif, vpr, and vpu). The gag gene encodes the core and matrix proteins, the pol gene encodes the viral enzymes, and the env gene encodes the envelope glycoproteins (Fig. 5.2). The accessory genes regulate the virus's ability to infect, replicate, and evade the host's immune response. This sophisticated structure and genome enable HIV to efficiently infect and replicate within human immune cells, particularly CD4+ T cells, leading to immune system deterioration and progression to AIDS without effective treatment. HIV-1 contains Vpu (viral protein U), while HIV-2 contains Vpx (viral protein X), with each playing a distinct role as shown in Table 5.1.

HIV-1 and HIV-2 are the two main types of the human immunodeficiency virus, each with distinct characteristics and geographical prevalence. HIV-1 is the more widespread and virulent of the two, responsible for the global pandemic (Hemelaar et al. 2006). It is divided into four groups: M (major), O (outlier), N (non-M, non-O), and P (pending), with Group M being the most prevalent (Mourez et al. 2013) and further subdivided into subtypes or clades (A, B, C, D, F, G, H, J, and K). These subtypes have different geographical distributions, with subtype B being most common in North America and Europe, while subtype C is predominant in sub-Saharan

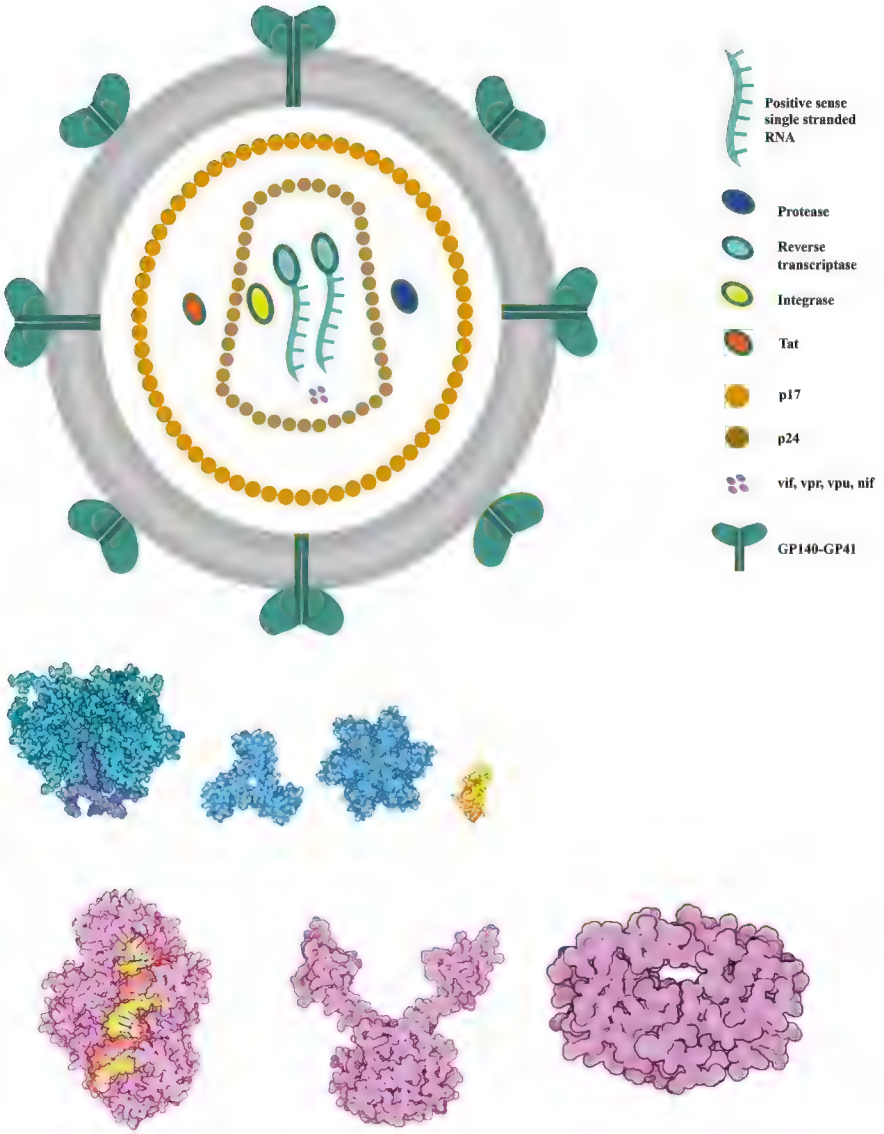

Fig. 5.1 Schematic and overall representation of HIV nucleocapsid (based on PDB-101 poster, RCSB.org). Entire viral assembly, Gp120 and Gp41 proteins, matrix proteins, capsid protein, nucleocapsid protein. Three main enzymes in the viral particles (1) RT, reverse transcriptase; (2) integrase, and (3) proteases (protein sizes—not on scale)

Africa and India (Korber et al. 2000; Brennan et al. 2008). HIV-2, on the other hand, is less transmissible and typically associated with a slower disease progression. It is primarily found in West Africa, though cases have been reported globally due to travel and migration. HIV-2 is classified into eight groups (A to H), but only groups

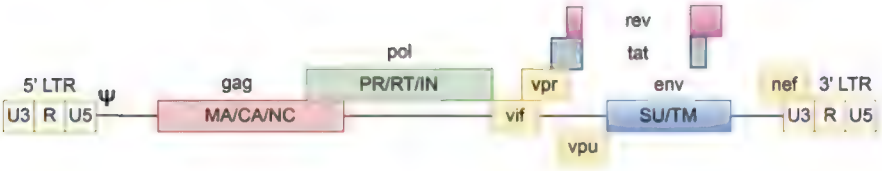

Fig. 5.2 Schematic illustrating the structure of HIV genome. The genome consists of three structural genes (gag, pol, env), four accessory genes (vif, vpr, vpu, and nef), and two regulatory genes (tat and rev)

Table 5.1 Function of HIV-1 and HIV-2 genes

Gene	Function (HIV1)	Function (HIV2)
gag	Core and matrix proteins	Core and matrix proteins
pol	Reverse transcriptase, integrase, protease	Reverse transcriptase, integrase, protease
env	Envelope glycoproteins (gp120, gp41)	Envelope glycoproteins (different sequences from HIV-1)
tat	Enhances transcription	Enhances transcription
rev	Regulates RNA export	Regulates RNA export
vif	Counteracts APOBEC3G	Counteracts APOBEC3G
vpr	Nuclear import of pre-integration complex	Nuclear import of pre-integration complex
vpu	CD4 degradation, virion release enhancement	Absent
vpx	Absent	Degrades SAMHD1
nef	Downregulates CD4 and MHC I	Similar function with sequence differences

A and B are epidemic, with the others being rare and less studied (de Silva et al. 2008). The genetic differences between HIV-1 and HIV-2 result in variations in their replication rates, pathogenicity, and response to antiretroviral therapy (ART). Understanding these differences is crucial for developing effective diagnostic, treatment, and prevention strategies tailored to the specific challenges posed by each type of HIV.

5.4 HIV Transmission and Epidemiology

HIV transmission primarily occurs through the exchange of body fluids such as blood, semen, vaginal and rectal fluids, and breast milk from an infected person to a healthy individual. The most common modes of transmission are unprotected sexual contact and sharing needles or syringes with an HIV-positive individual. HIV can also be transmitted from mother to child during pregnancy, childbirth, or breastfeeding, known as vertical transmission. Although less common, transmission can occur through blood transfusions, organ transplants, or exposure to contaminated

blood products, particularly in settings where screening protocols are inadequate. Preventive measures, such as using condoms, taking pre-exposure prophylaxis (PrEP), ensuring safe blood supply, and employing harm reduction strategies for intravenous drug users, significantly reduce the risk of transmission. Early diagnosis and antiretroviral therapy (ART) are also critical, as maintaining an undetectable viral load in an HIV-positive individual effectively eliminates the risk of sexual transmission. Public health education and awareness campaigns continue to play a vital role in preventing the spread of HIV and combating the stigma associated with the virus.

5.4.1 Life Cycle of HIV Within Host Cells

The primary target of HIV is CD4+ T cells. Following transmission, HIV establishes itself in mucosal tissues and subsequently spreads to lymphoid organs within a matter of days. By around day 10, the virus becomes detectable in the bloodstream, marking the onset of rapid viral spread over subsequent weeks. This proliferation often reaches its peak around day 30, coinciding with the emergence of detectable HIV antibody levels. This phase is typically when individuals are most infectious. Following this acute phase, the immune system exerts some level of control, leading to a "set point" where HIV replication stabilizes, often persisting for years. However, throughout this chronic phase, HIV continues to induce progressive depletion of CD4+ T cells and various immunological abnormalities through complex mechanisms. Over the course of several years, the persistent assault on the immune system by HIV leads to profound immunodeficiency, characterized by a decline in CD4+ T cell counts and a range of immunological dysfunctions. This immunocompromised state predisposes individuals to a spectrum of infectious and oncological complications that define acquired immunodeficiency syndrome.

5.4.2 Mechanism of HIV Infection

The mechanism of HIV infection involves several key steps that enable the virus to enter, replicate, and persist within the host's immune cells. HIV targets CD4+ T cells, macrophages, and dendritic cells, which express the CD4 receptor on their surfaces. The virus's envelope glycoprotein gp120 binds to the CD4 receptor. This binding induces a conformational change in gp120, allowing it to interact with a coreceptor, either CCR5 or CXCR4, on the host cell. The interaction with the coreceptor triggers further conformational changes, leading to the exposure of gp41, another viral glycoprotein. gp41 facilitates the fusion of the viral envelope with the host cell membrane, allowing the viral RNA and enzymes to enter the host cell. Once inside the host cell, HIV releases its RNA genome and the enzyme reverse

transcriptase. Reverse transcriptase converts the single-stranded viral RNA into double-stranded DNA. This process is error-prone, leading to a high mutation rate and contributing to the virus's ability to evade the immune system and develop drug resistance. The newly synthesized viral DNA is transported into the host cell's nucleus. HIV integrase, another viral enzyme, facilitates the integration of the viral DNA into the host cell's genome. This integrated viral DNA is known as a provirus. The provirus can remain latent for a period, making the infection chronic and difficult to eradicate. When the host cell is activated, the proviral DNA is transcribed into messenger RNA (mRNA) by the host's cellular machinery. The mRNA is then translated into viral proteins using the host cell's ribosomes. These proteins include structural components (such as Gag, Pol, and Env) and regulatory/accessory proteins (such as Tat, Rev., Nef, Vif, Vpr, and Vpu). Viral proteins and RNA genome assemble at the host cell membrane. The immature virion buds off from the host cell, acquiring its lipid envelope from the host cell membrane. After budding, the viral protease enzyme cleaves the Gag and Gag-Pol polyproteins into their functional components. This proteolytic processing results in the mature and infectious form of the virus, ready to infect new host cells (Fig. 5.3).

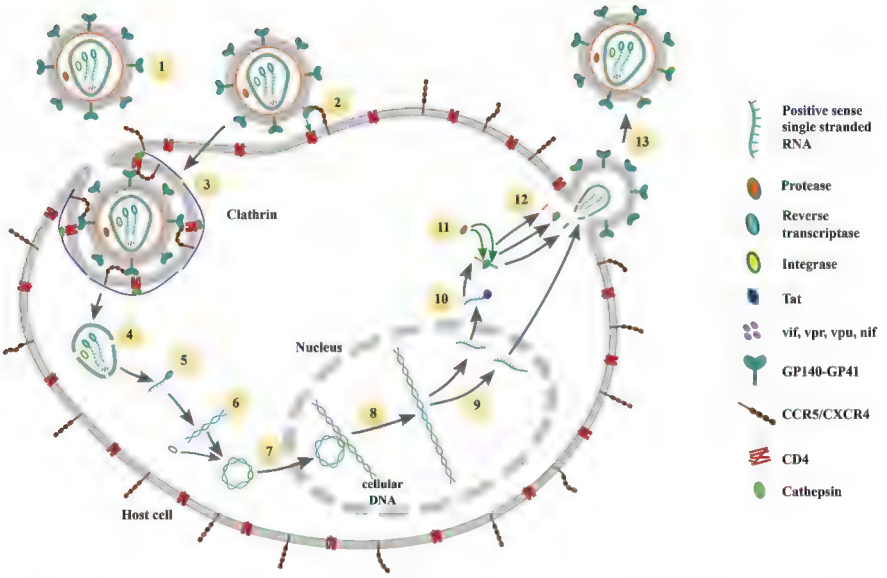

Fig. 5.3 Mechanism of HIV infection: (1) HIV containing a single copy of RNA; (2) Env-CD4 binding; (3) Fusion with host cell; (4) Uncoating; (5) Viral RNA release; (6) Reverse transcription of viral RNA; (7) Nuclear transport of pro-viral DNA; (8) Integration into host genome; (9) Transcription; (10) Viral RNA transport; (11) Translation of viral RNA; (12) Viral assembly

5.4.3 *Pathogenesis of HIV/AIDS*

The pathogenesis of HIV/AIDS involves the intricate interplay between viral replication and the host's immune response, resulting in the gradual deterioration of the immune system. Following initial infection, HIV targets and infects CD4+ T cells, macrophages, and dendritic cells, crucial components of the immune system. The virus's entry into these cells allows it to replicate rapidly, leading to a high viral load in the bloodstream during the acute phase of infection. Symptoms during this phase are often flu-like, accompanied by a temporary decline in CD4+ T cell count. As the immune system mounts a response, the viral load decreases, and CD4+ T cell levels partially recover, leading to the chronic phase of infection. During the chronic phase, HIV continues to replicate at lower levels, persistently infecting and depleting CD4+ T cells. The virus establishes latent reservoirs within resting CD4+ T cells and other tissues, evading both the immune response and antiretroviral therapy (ART). Over time, the continuous destruction of CD4+ T cells and the immune system's inability to replenish them lead to a gradual decline in immune function. The loss of CD4+ T cells impairs the body's ability to mount effective immune responses against opportunistic infections, hallmark conditions of AIDS (acquired immunodeficiency syndrome). Without treatment, the immune system weakens, leaving individuals vulnerable to opportunistic infections like pneumocystis, tuberculosis, and Kaposi's sarcoma. The progression from HIV infection to AIDS is marked by a significant drop in CD4+ T cell count (below 200 cells per microliter) or the occurrence of opportunistic infections.

5.5 Diagnosis of Human Immunodeficiency Virus (HIV) Infection

There are three stages of HIV infection: acute, chronic, and clinical. The first stage is the acute phase, which is marked by detectable viral nucleic acid and the p24 antigen in the blood (Fiebig et al. 2003). Host antibodies to HIV are not yet detectable, making the host seronegative. RNA levels peak around 6 weeks post-infection, while the CD4 T cell count decreases rapidly before showing a modest increase. Immunoglobulin (IgM) becomes detectable during first 2 weeks post-infection, followed by Immunoglobulin G (IgG) around 3–4 weeks post-infection (Bangalee et al. 2021). The second stage is the chronic phase, which begins with the detection of host antibodies to HIV, typically 6–8 weeks after infection. Seroconversion marks the start of this stage, with the host antibody response evolving and maturing over several years. HIV RNA levels remain stable, while CD4+ T cells decline gradually. The third and final stage is clinical AIDS, characterized by symptoms of immunodeficiency and opportunistic infections such as herpes, pneumonia, and fungal infections. Clinical AIDS is diagnosed when the CD4 T cell count falls below 200 cells/mm^3, accompanied by an increase in HIV RNA copies (Oni et al. 2013).

Table 5.2 Window period for the HIV test

Test	Window period (days)
Nucleic acid test (NAT)	10–33
Antigen/antibody lab test	18–45
Rapid antigen/antibody test	18–90
Antibody test	23–90

Source: https://www.cdc.gov/hiv/testing/index.html

HIV detection involves identifying host antibodies to virus proteins, RNA, or the virus itself. The main goal is to reduce the diagnostic window. Blood samples, such as plasma, serum, or dried spots, are preferred for HIV detection and can be stored long-term (Table 5.2). These samples also provide information on viral type, subtype, viral load, drug resistance, and the recency of infection. Urine or saliva tests are sensitive and specific but lack certain analytical capabilities compared to serological tests.

5.6 Serological Tests

5.6.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, also known as enzyme immunoassay (EIA), is a commonly used laboratory test for detecting and measuring antibodies. ELISA is also used for detecting HIV antibodies and/or p24 protein in the patient's sample (Rao et al. 1997). There are two types of ELISA: indirect ELISA and sandwich ELISA. In this method, HIV antigen and antibody immobilized plates are used to capture HIV antibodies or antigens in the sample. Secondary antibodies conjugated to enzymes are then used to detect the captured p24 antigen or HIV antibodies. ELISA is primarily used for screening patient's HIV antibodies mainly of the IgG subtype. Anti-HIV IgM is less sensitive and found in lower concentrations in the patient's serum. Indirect ELISA involves incubating the patient's serum with viral antigen and then detecting it using enzyme-linked secondary antibodies or enzyme-labeled antigens. As the disease progresses, the predictive value increases, leading to a higher false negative detection rate.

5.6.2 Western Blot (Immunoblot)

Western blot is used as a confirmatory test for HIV diagnosis before release of the result in case of any false positive. It is a sensitive and specific method that can detect antibodies to HIV in a patient's blood sample. The procedure involves separating proteins from the blood sample using gel electrophoresis based on their molecular weight and then transferring them to a membrane (nitrocellulose or

PVDF). The membrane is subsequently probed with specific antibodies that bind to HIV proteins, enabling their detection. Western blotting is a crucial tool in confirming HIV infection and is often used in conjunction with other tests for accurate diagnosis.

However, the Western blot method has limitations. While it was previously used to confirm ELISA results, it is no longer recommended due to its reduced reliability and slower diagnostic process. Other tests now offer enhanced accuracy and efficiency, leading to quicker and more reliable diagnosis.

5.6.3 Immunofluorescence Assay

The immunofluorescence assay (IFA) is another confirmatory test used to detect HIV infection. In this test the sample (serum or blood) is incubated with infected T cells expressing HIV antigen and uninfected cells as a control. Infected cells contain an antibody which binds to HIV antigen which is then conjugated to fluorescent molecules (fluorescein isothiocyanate) to emit light when exposed to UV radiation. Whether the sample is infected or not is determined by the degree and pattern of fluorescence. One of the US Food and Drug Administration (US-FDA)-approved confirmatory assays is fluorognost HIV-1.

The specificity of detection is higher when the test is done after seroconversion, but there is a higher chance of false negatives during the interval between infection and seroconversion. These tests are primarily designed to detect HIV-1 type M, subtype B infections and may not be sensitive to HIV-1 group O or HIV-2, potentially leading to false-negative results.

5.6.4 Rapid Diagnostic Test

Rapid HIV diagnostic tests are essential for situations requiring immediate results especially in difficult to reach populations in high-prevalence areas. These tests provide results in less than an hour, do not require highly skilled personnel, and can be used for various sample types such as plasma, urine, or oral secretions. Various rapid assay formats are available such as agglutination assays, solid-phase tests (ImmunoDOT), immunoconcentration devices, and immunochromatography. However, rapid tests have limitations as they primarily detect HIV antibodies rather than the p24 antigen, especially during primary HIV infections leading to false negative results. Despite this, rapid tests play a crucial role as initial screening steps, with results confirmed by standard laboratory HIV tests. They are particularly useful in emergency situations and for pregnant women during delivery, offering a convenient method of HIV diagnosis in areas with limited transportation and infrastructure. The positive predictive value of rapid tests increases in populations with high HIV prevalence, making them suitable for use in regions with a high burden.

When used in combination, rapid HIV tests can expedite result reporting, enhance result-specific counseling, and reduce false-positive results, especially in areas with low HIV prevalence.

5.6.5 Salivary and Urine Tests

Saliva is used as an alternative to blood tests for detecting HIV antibodies because it is simple, rapid, noninvasive, painless, and inexpensive. The OraQuick In-Home Test and the OraQuick Rapid Antibody Test (OraSure Technologies, Inc.) are a widely used diagnostic tests designed to detect antibodies to HIV-1 and HIV-2 in oral fluid or fingerstick blood samples. Approved by the FDA for both professional and over-the-counter use, this test provides results within 20 minutes, making it a convenient option for quick screening. It is administered in various settings outside traditional healthcare facilities, such as community health centers and at home. One of the main advantages of these tests is their ability to increase access to HIV testing among populations that might avoid traditional blood tests due to fear of needles, privacy concerns, or logistical barriers. However, like all antibody tests, it may not detect recent infections during the window period, so follow-up testing or confirmatory tests may be necessary for conclusive results.

Urine test for HIV infection is a non-invasive method used to screen for the presence of HIV antibodies. The test offers an alternative for individuals who may be uncomfortable with blood draws or fingersticks, enhancing the accessibility and acceptability of HIV testing. A urine sample collected from individuals is sent to a laboratory where it undergoes an enzyme immunoassay (EIA) to detect HIV antibodies. Similar to blood-based assays, results indicate whether HIV antibodies are present in the urine. Like other antibody tests, urine tests may not detect very recent HIV infections, as antibodies take time to develop after exposure. Positive results from urine tests typically require confirmation with blood tests to ensure accuracy.

Salivary and urine tests for HIV are not as commonly used as blood tests, but they provide a valuable option for increasing the reach of HIV screening programs, particularly in settings where traditional blood tests might be less feasible.

5.7 Direct Detection of HIV

5.7.1 Nucleic Acid Based Assays

HIV nucleic acid-based assays are crucial for amplifying HIV RNA from clinical samples, enabling accurate quantification and sequencing of the virus. These assays detect and measure HIV RNA levels, which are essential for diagnosing and managing HIV infection. One common technique is reverse transcription followed by

polymerase chain reaction (RT-PCR). In this method, HIV RNA is converted into complementary DNA (cDNA) using reverse transcriptase. The cDNA is then amplified through PCR, generating multiple copies of the DNA to enhance detection sensitivity. The amplified DNA is subsequently hybridized with complementary oligonucleotide molecules linked to an enzyme, resulting in a color change that allows for quantification (Rouet et al. 2008; Renault et al. 2022).

Another approach involves hybridization-based assays, where HIV RNA is captured by short complementary nucleic acid strands on a plate (Wang and Palmer 2018). In one variation, called branched DNA assay, branched oligonucleotides are used to capture RNA and enzyme simultaneously (Gleaves et al. 2002; Baumeister et al. 2012). The enzyme–nucleic acid complex produces a color change for quantification. Examples of assay using this method include the Quantiplex HIV-1 RNA (Erice et al. 2000). Another method for detecting HIV viral load is nucleic acid sequence-based amplification (NASBA). In contrast to standard ELISA antibody tests, NASBA with fluorescence correlation spectroscopy (FCS) is highly sensitive for the detection of HIV RNAs in plasma, even during the early stage of infection (Oehlenschläger et al. 1996). This technology allows for accurate and early diagnosis of HIV-1 infection. Monitoring the amplification reaction online with FCS helps estimate the initial HIV-1 RNA concentration and distinguish between positive and false-positive samples. NASBA with FCS is also valuable for assessing the effectiveness of anti-HIV treatments, especially in the early infection phase. This improved NASBA technology provides a more sensitive and accurate RNA detection method. Tests for viral load can be conducted on plasma, peripheral blood mononuclear cells (PBMCs), saliva, cerebrospinal fluid, and dried blood spots (DBS). DBS (dried blood spots), in particular, are gaining attention for their usefulness in remote areas with limited plasma transportation (Tang et al. 2016).

5.7.2 p24 Antigen Detection

The p24 antigen is a key HIV protein found in the viral core. It is detectable in blood shortly after infection (Gray et al. 2018), making p24 antigen assays valuable for diagnosis of early HIV infection. The p24 antigen detection test utilizes serological specimens to detect the p24 antigen through EIA. Although this test is less sensitive than HIV RNA assays, which have a sensitivity range of 10% to 95%, it has been integrated into fourth-generation HIV screening assays. These assays can detect both anti-HIV antibodies and the p24 antigen, making them more sensitive than tests that only detect antibodies. One such assay, the VIDAS HIV DUO Ultra, has been shown to shorten the diagnostic window by approximately 4 days (Weber et al. 2002). These advancements allow for earlier detection of HIV infection, facilitating prompt counseling and treatment of affected individuals.

5.7.3 *HIV Culture*

This HIV diagnostic test involves isolating the virus from various body fluids and tissues, including plasma, saliva, semen, tears, lymphoid tissues, and brain tissue. Although it is expensive, time consuming, and poses risks to lab workers, culture remains essential for research as it provides a way to study the live virus. For culturing, blood is collected with an anticoagulant and processed to isolate peripheral blood mononuclear cells (PBMCs), which are then co-cultured with cells from healthy donors or an indicator cell line to encourage virus growth. Cultures are maintained for 4 weeks and checked weekly for virus indicators, such as viral proteins or DNA. Despite its complexities, virus culture is indispensable for in-depth HIV research (Burgard et al. 1992). However, due to its high cost and labor-intensive nature compared to standard HIV antibody or nucleic acid-based assays, it is primarily used for therapeutic monitoring of patients in clinical trials who have undetectable level viral RNA levels by quantitative HIV RNA assays.

5.8 T-Cell Count Using Flow Cytometry

Infection with HIV causes a decline in T helper cells, which contain the CD4 surface protein. It is possible to identify these cells by staining them with antibodies to CD4 and analyzing them with a flow cytometer. The results are typically expressed as a percentage of peripheral blood mononuclear cells (PBMC) that are CD4 positive. In a healthy individual, the percentage of CD4 positive cells ranges from 25% to 65% of all T cells. In patients with HIV, the number of CD4 T cells decreases by approximately 80 cells/μl each year until it reaches 400–500 cells/μl. The CD8+ cells in the body increase during acute infection and return to normal within a month of the infection (Collins et al. 2020).

5.9 Emerging Point-of-Care Molecular Tests for HIV

5.9.1 *Paper and Flexible Material-Based Assays*

Paper and flexible material-based assays for HIV diagnosis have emerged as innovative solutions to enhance accessibility, affordability, and rapidity in HIV testing, especially in resource-limited settings. These assays such as lateral flow assays (LFs) and microfluidic paper-based analytical devices (μPADs), utilize simple materials like cellulose and nitrocellulose paper to create low-cost diagnostic tools (Martín González et al. 1988; Tang et al. 2016). By utilizing capillary action, these assays can efficiently transport samples through test zones treated with specific reagents, resulting in detectable colorimetric changes upon interaction with HIV

antibodies or reagents. Their ease of use, minimum requirements for specialized equipment, and quick turnaround times make them ideal for point-of-care (PoC) testing. Additionally, advancement in patterning techniques and integration with digital technologies, such as smartphone-based result analysis, further enhance their functionality and user-friendliness. These assays not only facilitate early and accurate HIV detection but also hold the potential to significantly improve public health outcomes by enabling timely treatment and reducing transmission rates. Despite challenges related to sensitivity, specificity, and reagent stability, ongoing research and technological innovation continuously refining and expanding the capabilities of paper and flexible material-based HIV diagnostics.

5.9.2 Plasmonic Nanoparticles and Photonic Crystal-Based Platforms

Surface plasmon resonance (SPR) and photonic crystals (PC) are emerging methods in biosensing, primarily used for virus detection. Plasmonic nanoparticles typically composed of metals like gold or silver, exhibit unique optical properties due to localized surface plasmon resonance (LSPR). LSPR detects changes in the refractive index within the electromagnetic field at the nanoscale in noble materials, providing highly responsive sensing. On the other hand, photonic crystals (PC)-based biosensors are optical materials which offer rapid and label-free detection by monitoring changes in dielectric permittivity during molecular interactions. These methods have demonstrated potential for HIV detection Shafiee et al. 2014, but they encounter challenges such as equipment costs and the requirement for further development to handle clinical samples like blood, saliva, and urine.

5.10 Other HIV Diagnostic Methods

The Atomo HIV self-tests (HIVST) represents a significant advancement in the accessibility and convenience of HIV testing. Designed for home use, this test allows individuals to privately and easily check their HIV status without the need for a healthcare professional. The Atomo HIV self-test typically involves a simple procedure, such as a finger-prick to collect a small blood sample or a swab of oral fluids. The test kit includes all necessary components and provides clear instructions, ensuring ease of use. Results are usually available within 20–40 minutes, offering a quick turnaround. By empowering individuals to take control of their health, the Atomo HIV self-test helps reduce the stigma associated with HIV testing and encourages more people to know their status. Early detection through such accessible testing can lead to timely treatment and better management of the disease, ultimately contributing to improved public health outcomes.

5.11 Challenges and Future Directions

Staying abreast of HIV evolution, drug resistance development, and global spread is increasingly important to ensure accurate diagnostic tests and antiretroviral drugs are accessible in high prevalence areas. This is essential for reducing transmission rates and effectively managing the challenges posed by the virus. The primary challenges in diagnostic methods include nucleic acid testing being the most tests and emerging technologies offer hope for easier diagnosis in resource-limited settings, but their effectiveness and accessibility are still under development.

In developed countries, individuals living with HIV typically have access to comprehensive healthcare services, including specialist care and ongoing monitoring. However, this model is not feasible in resource-limited settings (RLS) where a decentralized approach to HIV testing proves more effective. It is imperative that diagnostic tools in these settings meet specific criteria, including accuracy, affordability, rapidity, and simplicity of administration, robustness, and operation without electricity, refrigerated reagents, or complex equipment.

Diagnostic tests have their advantages, such as nucleic acid testing offering higher accuracy and reliability and the newer ELISA capable of detecting viral proteins, antibodies, and p24 antigens simultaneously. However, these tests are limited to hospitals, clinics, and laboratories common only in developed countries. In resource-limited settings, NAT and ELISA are not ideal options due to limited access to clinic access. Implementing dried blood sample (DBS) testing, faces challenges such as reduced sample recovery, low sensitivity, and potential biomarker decomposition after prolonged storage. Point-of-care (PoC) rapid tests provide results within 30 minutes and are portable, but quality assurance by experts is necessary to monitor test efficiency and performance and determine the need for confirmatory tests.

In infants, HIV detection using antibodies is challenging until 18 months due to maternal antibodies masking their true HIV status. Standard practice involves administering antiretroviral therapy (ART) at birth and conducting NAT tests at 2 and 4 weeks old, with two negative results confirming no HIV transmission. Monitoring during breastfeeding is essential due to the risk of HIV transmission through breast milk. In RLS, HIV status is often determined based on clinical symptoms, or dried blood samples sent to laboratories and the use of ultra-sensitive p24 assays. In adults, it takes about 3 weeks to 6 months to detect anti-HIV antibodies before seroconversion, but HIV is highly transmissible during the acute phase of infection, where viral load increases and antibodies may not be detectable. Accurate distinction between recent and long-standing infections is crucial for understanding transmission patterns and guiding public health interventions. Conventional antibody detection tests cannot achieve this distinction. Serological testing algorithms like serological testing algorithm for recent HIV seroconversion (STARHS) can identify recently occurred infections. Various incidence testing methods such as BED (BED stands for HIV-1 subtypes B,E and D is an enzymatic immunoassay to measure the amount of IgG that is targeting the immunodominant region of HIV-1

gp41 by using BED capture), avidity, IDE-V3, and detuned assays offer different approaches to estimating recency of infection. Challenges arise in calibrating and validating these assays, including specimen panel representation, uncertainty in seroconversion dates, and variations in test sensitivity and specificity. HIV's genetic diversity poses challenges for diagnostic assays due to variations in prevalence, incidence rates, transmission patterns, and circulating recombinant forms (CRFs) across regions. Strain variants lacking specific markers or caring different ones can lead to inaccurate results in antibody and nucleic acid tests. Region-specific reagents and targeting highly conserved markers or gene sequence are needed to address discordance in different results from assays conducted in different regions.

Vaccine recipients may produce antibodies similar to those induced by natural HIV infection, leading to false-positive results and challenges in differentiating between vaccine-induced and virus-induced antibodies. This misidentification can have significant effects on public health including

- Inability to assess the true HIV status in vaccine recipients.
- Difficulty in deterring exposure timing relative to vaccination.
- Rejection of blood donations from vaccinated individuals who are not infected.
- Unnecessary anxiety and medical care for falsely identified HIV-positive individuals.

The lower sensitivity and lengthy turnaround time of current diagnostic tests, make them impractical for emergency situations like transplants. HIV weakens the immune system, making a person susceptible to opportunistic infections such as tuberculosis (TB), malaria, hepatitis C virus (HCV) and hepatitis B virus (HBV). A majority of HIV-positive individuals die from TB. Coinfections not only raise the mortality rates but also complicate HIV testing, necessitating diagnostics that can accurately account for them. Traditional TB diagnosis methods such as sputum smear microscopy and culture are often inaccurate and slow. While there are advancements in NAT platforms for rapid TB detection, their accuracy and feasibility in RLS remain uncertain due to high costs per test and logistical challenges in deploying instruments to remote areas.

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Chapter 6
Recent Progress in Molecular Diagnostics of Hepatitis Viruses

Adila Khanam, D. P. Sinha, and Jawed Iqbal

Abstract Viral hepatitis is a global health issue that causes liver inflammation and can lead to acute or chronic liver problems if not treated effectively. Early detection of viral hepatitis is crucial for medical intervention, virus control, and antiviral medications, which might be helpful to improve patient outcomes. Different genomic structures are found in hepatitis viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV), that affect their pathogenicity and epidemiology. Different nucleic acid amplification-based techniques are available for the detection of hepatitis viruses with high specificity and sensitivity. Fluorescence in situ hybridization (FISH) can detect hepatitis A, B, and C viruses in tissues. Moreover, non-amplification-based molecular diagnostics, like next-generation sequencing (NGS) and enzyme-linked immunosorbent assay (ELISA), provide alternative approaches for detecting sequence viral genomes and viral proteins, respectively. Recent clustered regularly interspaced short palindromic repeat (CRISPR)-based systems offer advanced diagnostic capabilities for the quick detection and management of viral hepatitis viruses specially HCV and HBV with high sensitivity, and specificity which have the potential to dramatically improve healthcare system. This book chapter summarizes the recent advancement in the different techniques which are being used or in progress to be used for the detection of hepatitis viruses.

Keywords Hepatitis · Molecular diagnosis · Real-time PCR · ELISA · Next-generation sequencing and CRISPR-based detection

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6.1 Introduction

"Viral hepatitis" refers to liver inflammation caused by a virus. Currently, there are five viruses that particularly attack the liver (hepatitis A, B, C, D, and E) (Webb et al. 2020). Viral hepatitis is a global health concern with increasing deaths. Despite some progress, the global response is insufficient. The majority portion of individuals with hepatitis chronic B or C persists as undiagnosed and receive inadequate antiviral therapy or treatment. The result comes short of the global goal of eliminating viral hepatitis by 2030. Access to prevention, diagnosis, and treatment varies by area, prompting immediate action to save millions of lives and avoid future infections and cancer cases by 2026 (World Health Organization 2023). When serological testing are inconclusive, molecular diagnostics can help diagnose viral hepatitis infections. Nucleic acid tests are used to manage these viruses, specifically B and C, by detecting viral levels in blood or plasma and monitoring drug responses. Assays to assess viral load in liver tissue have also been developed (Zanella et al. 2002; Lok and McMahon 2009). Nucleic acid diagnostic tests are crucial in modern medical diagnostics, with Nucleic acid sequence-based amplification (NASBA), polymerase chain reaction (PCR), and real-time PCR which are being frequently used for virus detection, genotyping, and quantification. Automation has improved efficiency, reduced test time and contamination risks while enhancing performance and lowering detection limits (Cobo 2012). PCR-based molecular diagnosis is a cornerstone in modern medical diagnostics, particularly in the detection and quantification of viral genomes. PCR is a classical target amplification method that offers unparalleled sensitivity and specificity. Detection of PCR amplicons relies on hybridization to immobilized oligonucleotide probes and enzymatic reactions. Quantification in PCR-based assays is achieved through competitive amplification with a known synthetic standard, facilitating accurate measurement of viral load. This molecular diagnostic approach has revolutionized the field, providing clinicians with precise tools for early detection, treatment response monitoring, and therapeutic interventions (Chevaliez et al. 2013). Hepatitis diagnosis involves laboratory tests to detect elevated enzymes like aspartate aminotransferase (AST) and alanine aminotransferase (ALT), along with markers like total bilirubin and specific viral antigens. Chronic hepatitis requires persistent serological markers for over six months. Molecular techniques like RT-qPCR detect viral nucleic acids. In blood tests, liver enzyme levels are crucial in diagnosis, and a liver biopsy confirms severity and stage of inflammation and additional tests like markers for fibrosis, ultrasound, endoscopy, and monitoring alpha-fetoprotein (AFP) levels aid in early detection of liver cancer and assess liver damage and complications (Prasidthrathsint and Stapleton 2019).

6.2 Viral Hepatitis and Disease Progression

Globally, viral hepatitis causes millions of deaths and chronic illnesses (World Health Organization 2016). Hepatitis B virus (HBV) infection results are made worse by hepatitis D virus (HDV). Most acute infections are caused by the hepatitis E virus (HEV). The World Health Organization (WHO) established a virus eradication campaign as a result of the harm that they together pose to public health (Wedemeyer and Manns 2010). Liver disease progression can be caused by many ways among which most common reasons are alcohol, overweight, viral hepatitis, and autoimmune liver conditions. In some people, extra fat in the liver may cause liver damage and if the damage is not recovered then it may cause an increase in inflammation in the liver. Further, scarring in the liver may be developed and with time it can lead to severe scarring (cirrhosis) and eventually hepatocellular carcinoma (Fig. 6.1).

HEV and hepatitis A virus (HAV) do not usually develop into a chronic disease, unlike HCV and HBV, and are not related to liver cancer. However, it can lead to serious illness and even death. For example, chronic HEV infection raises the chance of serious liver failure, stillbirth, or death. The epidemiology of both illnesses is significant and differs widely by place as a result of many risk factors and vaccination programs, such as unequal access to hygienic conditions and contact with pigs. There are more susceptible people and major outbreaks occurring in many areas due to improved sanitation and a decrease in other frequent dangers. This has also decreased the seroprevalence of protective antibodies (Costa-Mattioli

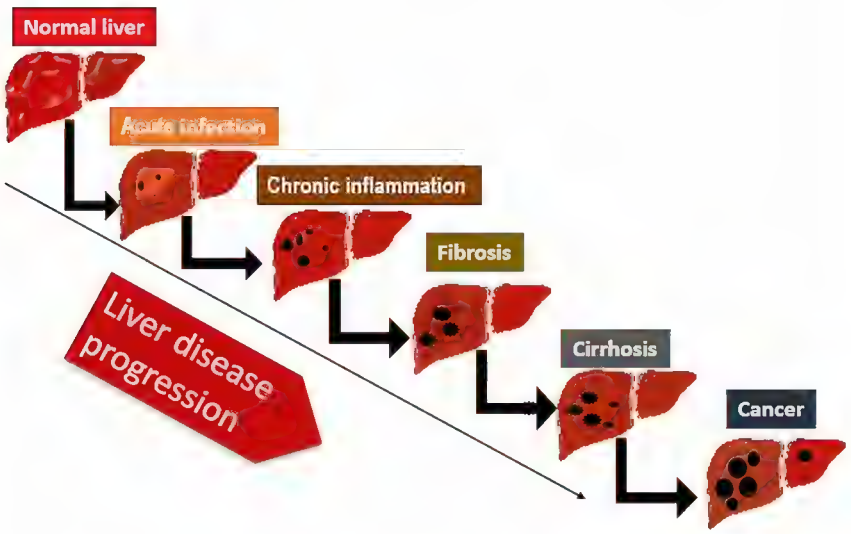

Fig. 6.1 Stages of liver disease progression infected by hepatitis viruses

et al. 2003; Kamar et al. 2017). The National Hepatitis C Program (NVHCP), introduced by Indian government in 2018, aims to prevent and treat hepatitis A, B, C, and E. Free screening, diagnosis, treatment, and referral services are offered (Kumar et al. 2015). Prioritizing a few key areas is necessary to eradicate HBV. The national immunization schedule of India includes a universal HBV dose at birth, which is the primary preventive approach for preventing any infection. However, its wider implementation is necessary (Premkumar and Chawla 2021). Like hepatitis A, no specific treatment is available for hepatitis E in India; instead, we should practice good hygiene and drink only clean water to reduce the burden of hepatitis E. Vaccination has proven ineffective and recombinant vaccines are currently being developed for frequent travelers and pregnant women (Kamar et al. 2017). To detect 30% of patients with chronic viral hepatitis B and C by 2020 and 90% by 2030, equity and accessibility of diagnostic and treatment services must be improved. Currently, only 10% and 19% of patients with chronic hepatitis C are aware of their status, compared to a global goal of 80% treatment coverage by 2030 (World Health Organization 2018; Hutin et al. 2018). Table 6.1 highlights different hepatitis virus's variants and their mode of detection.

6.3 Hepatitis Viruses

6.3.1 Hepatitis A Virus

Hepatitis A virus (HAV) is a non-enveloped single-stranded RNA virus that belongs to the Picornaviridae family and is the only species of the Hepatovirus genus that can infect humans (Ping et al. 1988). Its virion size is around 27 nm and its genome is 7.5 kb. The viral polyprotein is encoded by a single open reading frame (ORF) in the genome. This polyprotein is broken post-translationally to produce capsid and non-structural proteins (Fig. 6.2).

Genetic variety has been uncovered by sequencing investigations, which have defined seven distinct HAV genotypes. Only these four genotypes—I, II, III, and VII—have been connected to infections in humans. A capsid is assembled from mainly of four proteins: VP1, VP2, VP3, and VP4. VPs 1 and 3 are found on the outside and are required for receptor binding and immune response activation. Acute HAV infection typically manifests after an incubation period of 14–40 days, often asymptomatic in children. Shedding of the virus in feces precedes symptomatic illness, with the appearance of IgM anti-HAV antibodies diagnostic of acute infection. Long-lasting IgG anti-HAV antibodies indicate recovery or successful immunization post-vaccination (Costa-Mattioli et al. 2003; Ping et al. 1988; Prasidthrathsint and Stapleton 2019).

Table 6.1 Hepatitis viruses and their variants and genomes

Variant	Family	Genus	Genome Structure	Genome Length	Genome Organization	Diagnosis	References
Hepatitis A	Picornaviridae	Hepatovirus	ssRNA(+)	~7.5 kb	Linear	Detection of IgM antibodies in serum during acute infection	Prasidthrathsint and Stapleton (2019)
Hepatitis B	Hepadnaviridae	Hepatovirus	Partial dsDNA	~3.2 kb	Circular	HBsAg, anti-HBc, anti-HBs antibodies, and HBV DNA had been detected	Weber et al. (1999)
Hepatitis C	Flaviviridae	Hepacivirus	ssRNA(+)	~9.6 kb	Linear	Detection of HCV antibodies, HCV RNA by PCR	Lok (2007)
Hepatitis D	Orthohepadnaviridae	Deltavirus	ssRNA(−)	~1.7 kb	Circular	Detection of anti-HDV antibodies, HDV RNA by PCR	Wedemeyer and Manns (2010)
Hepatitis E	Hepeviridae	Orthohepevirus	ssRNA(+)	~7.2 kb	Linear	Detection of IgM antibodies in serum during acute infection	Binderman (2018)

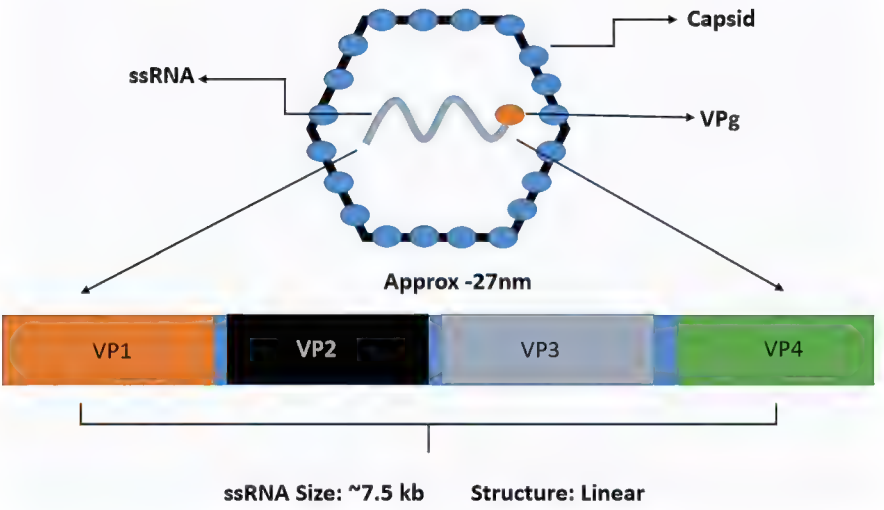

Fig. 6.2 Genomic structure of the hepatitis A virus, which is an icosahedral virus with a single-stranded RNA genome that encodes a polyprotein that is processed into structural and non-structural proteins

6.3.2 Hepatitis B Virus

HBV is a member of the Hepadnaviridae family. The HBV genome is a partially double-stranded, circular DNA molecule of approximately 3.2 kb in length (Seeger and Mason 2000). Its genome consists of four overlapping open reading frames (ORFs): S, C, P, and X (Newbold et al. 1995) (Fig. 6.3). S ORF (surface antigen) codes for the surface proteins: Small (S), Medium (M), and Large (L) envelope proteins (Bruss 2007). C ORF (core protein) codes for the core protein (HBcAg) which forms the nucleocapsid (Zlotnick et al. 2015). P ORF (polymerase) codes for the viral polymerase (Pol) which is essential for viral replication (Summers and Mason 1982). X ORF (X Protein) codes for the X protein(HBx), which regulates viral replication and has oncogenic properties (Levrero and Zucman-Rossi 2016).

Regulatory elements include promoters, enhancers, and the epsilon (ε) sequence involved in RNA packaging (Newbold et al. 1995). Replication strategy involves reverse transcription of an RNA intermediate (Summers and Mason 1982). Diagnostic tools for HBV are crucial for accurate detection andmanagement. Serological tests, such as the HBsAg and anti-HBc tests, are commonly used for screening and diagnosis, employing methods like enzyme immunoassay (EIA) and chemiluminescent immunoassay (CLIA) (Sharma et al. 2017). Molecular tests, such as PCR for HBV DNA and genotyping/sequencing for genotype determination, are essential for viral load measurement, treatment monitoring, and detecting drug resistance mutations (Lok and McMahon 2009; Durantel and Zoulim 2009). In resource-limited settings, rapid diagnostic tests (RDTs) based on lateral flow

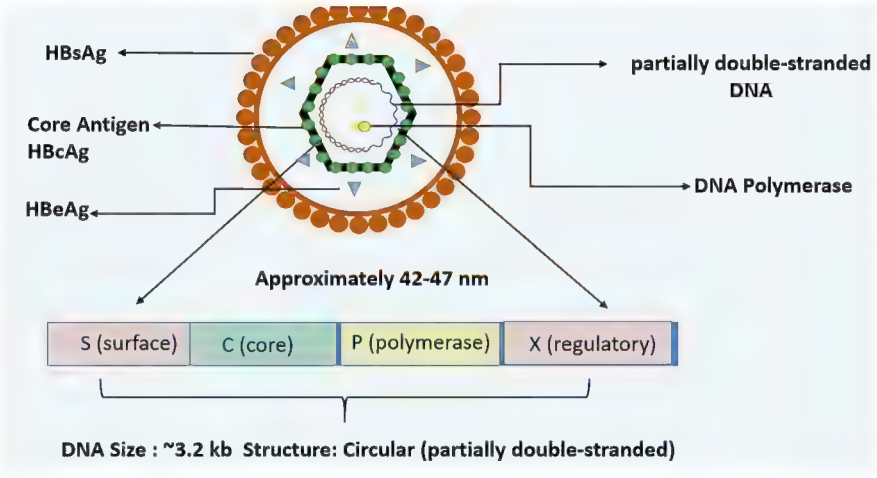

Fig. 6.3 Genomic structure of HBV

immunoassays are employed (Liaw and Chu 2009). These tools play a vital role in global efforts to diagnose HBV infections promptly, allowing for effective management and treatment strategies.

6.3.3 Hepatitis C Virus

Hepatitis C virus (HCV) is an enveloped RNA virus, approximately 60 nm in diameter, classified within the Flaviviridae family and its own Hepacivirus genus. The virus possesses a 9.6 kb single-stranded RNA genome, containing a single open reading frame (ORF) bordered by untranslated regions (UTRs) that control the translation and replication of the viral genome (Fig. 6.4).

The encoded polyprotein undergoes cleavage into structural proteins and non-structural proteins (NS) by host and viral proteases. HCV has both structural and non-structural proteins that are crucial for its life cycle and pathogenesis. The core protein forms the nucleocapsid, while envelope proteins facilitate entry into host cells. Non-structural proteins, such as NS3, play a role in viral replication and evasion of host immune responses, these proteins are essential for developing targeted therapies against HCV (Zein 2000). Transmission of HCV occurs primarily through parenteral routes, frequently resulting in a chronic infection in approximately 80% of cases. Diagnosis involves enzyme immunoassay (EIA) testing for antibodies and detection of HCV RNA via reverse transcription PCR (RT-PCR). Management includes treatment with pegylated interferon alpha combined with ribavirin, where the treatment duration depends on the virus genotype-6 months for genotypes 2 and 3, and 12 months for genotypes 1 and 4 (Lok 2007). Genotyping is essential due to significant nucleotide diversity among HCV strains, leading to seven main

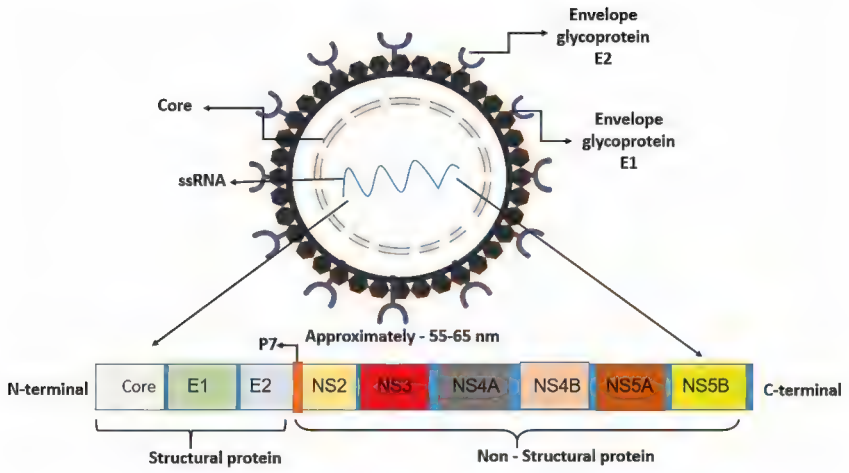

Fig. 6.4 Structure of HCV and its genome encodes for a large polyprotein that is processed into structural proteins (core, E1, E2, and P7) and nonstructural proteins (NS2-NS5B), which are required for viral replication and assembly

genotypes and over 70 subtypes. This genetic diversity is attributed to the error-prone nature of the RNA polymerase, which generates a spectrum of closely related genetic variants or quasispecies within an individual (Carman et al. 2005; Rong et al. 2010). The virus has an incubation period of 1–6 months, and unlike HBV, HCV more commonly spreads through non-sexual and non-vertical transmission methods.

6.3.4 Hepatitis D Virus

HDV infection is associated with severe forms of liver disease, including cirrhosis and hepatocellular carcinoma. It affects an estimated 15–20 million people globally (Wedemeyer and Manns 2010). HDV is a small, circular RNA virus approximately 1.7 kb in length (Fig. 6.5). It has a single ORF encoding two proteins: small and large delta antigens (HDAg-S and HDAg-L) (Hughes et al. 2011; Vieira et al. 2017). The small delta antigen (HDAg-S) is required for HDV RNA replication. The large delta antigen (HDAg-L) is involved in viral assembly and is essential for HDV virion formation (Hughes et al. 2011; Vieira et al. 2017). HDV utilizes host RNA polymerase II for replication. The replication process involves a rolling-circle mechanism (Hughes et al. 2011; Vieira et al. 2017). HDV particles are enveloped and are about 36 nm in diameter. The virus uses HBV envelope proteins (HBsAg) for its envelope. The nucleocapsid is composed of HDAg and the HDV genome (Hughes et al. 2011; Vieira et al. 2017). HDV infection can cause serious liver damage compared to HBV mono-infection. Mechanisms include direct cytopathic

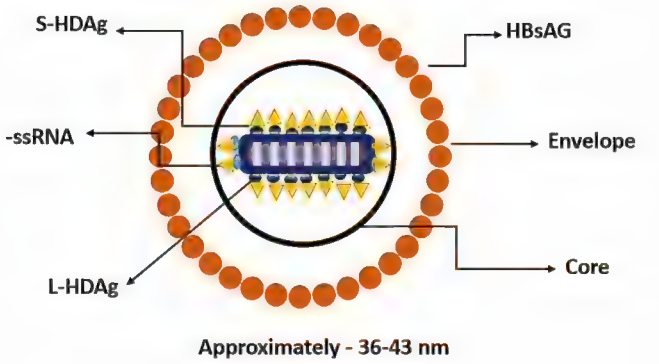

Fig. 6.5 Genomic structure of hepatitis D virus. It is a small and spherical, encapsulated virus with a diameter of 36–43 nm, that needs the hepatitis B virus (HBV) surface antigen (HBsAg) to build its envelope and proliferate, making it a satellite virus that relies on HBV for reproduction

effects and immune-mediated mechanisms (Hughes et al. 2011; Vieira et al. 2017). Pegylated interferon alpha is the standard treatment, although it has limited efficacy. Novel therapies under investigation include lonafarnib, bulevirtide, and combinations with entry inhibitors (Erhardt et al. 2006; Gençdal and Yurdaydin 2021). HDV infection is associated with an increased risk of liver-related morbidity and mortality. Continuous investigations are crucial for understanding the natural history of the disease (Heidrich et al. 2014; Hughes et al. 2011). HDV RNA PCR detects HDV RNA in serum or liver tissue using real-time PCR (qPCR) or quantitative PCR (qPCR) methods. This diagnostic tool is essential for measuring viral load and monitoring treatment response in hepatitis D patients (Vieira et al. 2017). Serological tests for HDV include anti-HDV IgM and IgG tests, which detect antibodies against HDV antigens. These tests are typically conducted using enzyme immunoassay (EIA) or chemiluminescent immunoassay (CLIA) methods. They are crucial for diagnosing HDV infection and determining the stage of infection (Vieira et al. 2017).

6.3.5 Hepatitis E Virus

Hepatitis E virus (HEV) is a small, non-enveloped RNA virus belonging to the family Hepeviridae. It possesses a single-stranded, positive-sense RNA genome of around 7.2 kb (Smith et al. 2014). The genome of HEV contains three open reading frames (ORFs): ORF1, ORF2, and ORF3 (Fig. 6.6). ORF1 encodes non-structural proteins involved in viral replication. ORF2 encodes the capsid protein, which is important for virus structure and immune recognition. ORF3 encodes a small multifunctional protein involved in virus egress and pathogenesis (Smith et al. 2014). HEV is typically spread by the fecal–oral route, which involves contaminated water

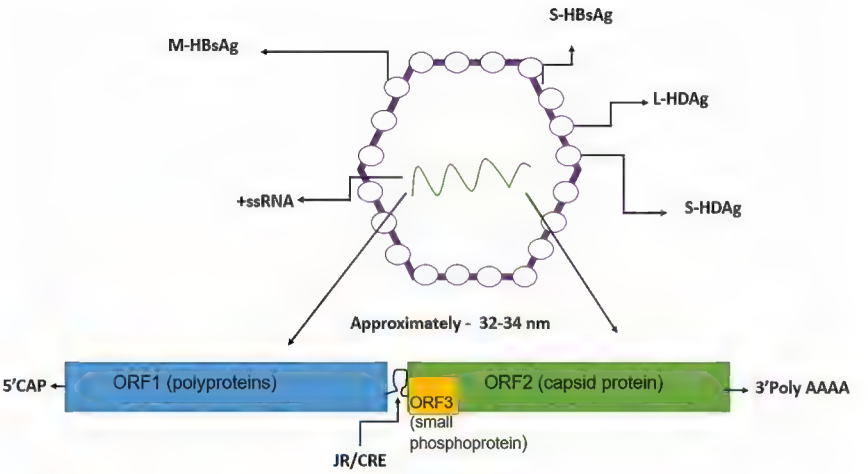

Fig. 6.6 Structure of HEV virus with a 32–34 nm diameter capsid. It is a positive-sense RNA that encodes three open reading frames (ORFs) responsible for viral replication, capsid protein synthesis, and protein processing. ORF1 encodes polyproteins, which are then processed into different enzymes. ORF2 codes for capsid protein and structural protein 2, whereas ORF3 codes for multifactorial protein, which is then converted into presumptive capsid protein. A junctional region joins ORF1 and ORF2/ORF3, which contains a stem loop sequence and two cis-reactive elements. The poly-adenosine tail sequence (poly-AAA) can be found at the 3′ end (Cao and Meng 2012)

or food. In endemic regions, HEV genotypes 1 and 2 are associated with large outbreaks due to poor sanitation and hygiene. Genotypes 3 and 4 are zoonotic and are transmitted from animals, particularly pigs to humans (Kamar et al. 2017). HEV infection typically causes an acute self-limiting hepatitis, similar to hepatitis A. In immunocompromised individuals and pregnant women, HEV infection can lead to chronic hepatitis and severe complications (Kamar et al. 2017). Serological tests detect anti-HEV IgM and IgG antibodies, indicating recent or past infection. Molecular methods such as reverse transcription polymerase chain reaction (RT-qPCR) detect HEV RNA in serum or stool (Kamar et al. 2017). There is no specific antiviral therapy for HEV infection. Prevention focuses on improving sanitation and hygiene, especially in endemic areas, and ensuring safe drinking water and food (Kamar et al. 2017).

HEV exhibits genetic diversity and is classified into different genotypes and subtypes based on sequence analysis. Genotypes 1 and 2 primarily infect humans and are associated with large outbreaks in endemic regions. Genotypes 3 and 4 are zoonotic and are transmitted from animals, particularly pigs, to humans (Smith et al. 2014; Lu et al. 2006). HEV shows evidence of recombination between different genotypes and with other Hepeviridae family members. Recombination contributes to the genetic diversity and evolution of HEV (Smith et al. 2014).

6.4 Polymerase Chain Reaction-Based Molecular Diagnosis

PCR techniques have revolutionized the field of virology, particularly in the diagnosis of viral hepatitis, including hepatitis B, C, D, and E viruses. PCR allows for the sensitive, specific, and quantitative detection of viral nucleic acids (Tang et al. 2013) (Table 6.2). Real-time PCR (qPCR) is the most commonly used PCR technique in clinical settings due to its ability to provide rapid results and quantify viral load. It works by detecting the fluorescence emitted during the reaction as the PCR product accumulates in real time (Vermehren et al. 2008). This method is advantageous for monitoring viral replication and assessing treatment response in hepatitis patients. PCR can be performed using different methods, each with specific applications depending on the viral target and diagnostic requirements. Nested PCR involves two rounds of PCR amplification and is particularly useful for detecting low-level infections (Chauhan et al. 2009). Multiplex PCR allows for the simultaneous amplification of multiple targets in a single reaction, which is beneficial for detecting co-infections or multiple virus strains (Shuber et al. 1995). Quantitative reverse transcription PCR (RT-qPCR) is used for detecting RNA viruses, such as HCV, by first converting RNA into complementary DNA (cDNA) using reverse transcriptase enzyme (Fig. 6.7).

These techniques enable the detection of low viral loads and the differentiation of viral genotypes, which are critical for guiding treatment decisions and managing patient care (Vermehren et al. 2008). HBV diagnosis via PCR typically involves monitoring the covalently closed circular DNA (ccDNA) of HBV in hepatocytes, which is the most precise method for assessing infected hepatocytes. However, this approach requires invasive procedures and is not commonly used in diagnostics. An alternative is quantifying HBV DNA levels in serum, a less invasive method. This serves as a substitute for cccDNA detection and is in line with the recommendations of the Taormina Group (Raimondo et al. 2008). The genetic segments of hepatitis viruses, particularly HBV and HCV, are key targets for PCR-based diagnostics. For instance, HBV has a partially double-stranded DNA genome that serves as a template for PCR amplification, allowing quantification of HBV DNA levels in the

Table 6.2 PCR components and its function

Component	Description
Template DNA	Contains the specific region to be amplified
Primers	Short single strands of DNA (oligonucleotides) complementary to each strand of the target region, both forward and reverse primers are required
Free nucleotide bases	Building blocks for new DNA strand synthesis
DNA polymerase	Enzyme that carries out DNA replication; thermostable analogs like Taq polymerase are used due to their resistance to the heating and cooling cycles of PCR

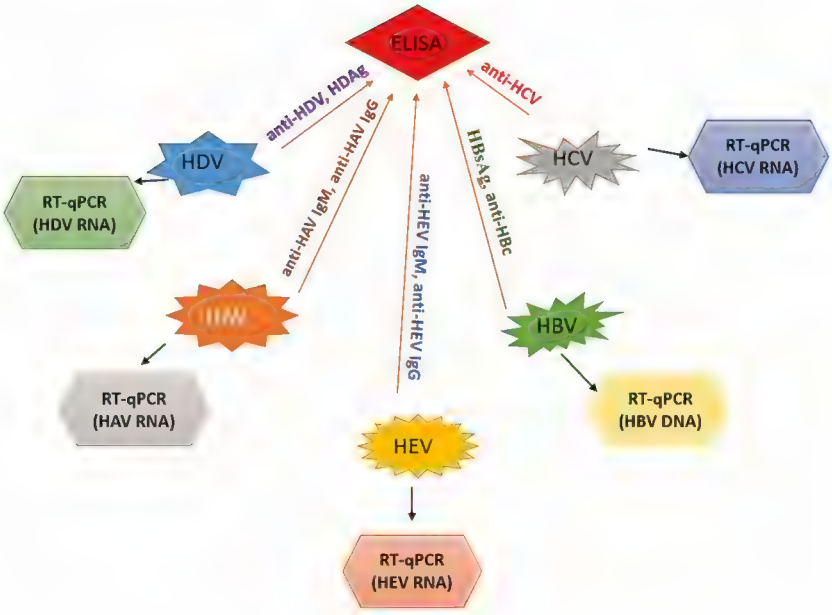

Fig. 6.7 Diagnostic methods for various hepatitis viruses. Enzyme-linked immunosorbent assay (ELISA) is a popular method for detecting these viruses. Other specialized tests include PCR and RT-qPCR for viral RNA or DNA quantification liver function tests, and serological testing for particular antibodies such as anti-HAV IgM/IgG, anti-HBV, anti-HCV, anti-HDV, and anti-HEV. These procedures help to diagnose and manage hepatitis infections

blood (Ganem and Prince 2004). Similarly, HCV is an RNA virus, and its RNA genome can be amplified using RT-qPCR, enabling quantification of HCV RNA levels (Vermehren et al. 2008). These PCR assays are essential for monitoring viral load and assessing treatment response in chronic hepatitis patients. The use of qPCR in hepatitis diagnosis has been extensively studied and validated, providing accurate and reliable results that contribute to improved clinical outcomes and patient management (Chauhan et al. 2009) (Fig. 6.7). Investigated the application of qPCR in detecting human coronaviruses in respiratory infections among children, showcasing the versatility of qPCR in diagnosing viral infections beyond hepatitis. These studies collectively underscore the significance of qPCR-based diagnostics in viral hepatitis and other infectious diseases, facilitating early detection, treatment monitoring, and epidemiological surveillance. There are some techniques which are derived from PCR technology and known as in vitro target amplification techniques (Table 6.3).

Table 6.3 PCR technique for detection of viral hepatitis

PCR technique	Hepatitis variant applicability	Advantages	Disadvantages	Time to detect	References
Conventional PCR	HBV, HCV, HEV	Simple and widely used	Limited sensitivity and specificity	Hours	Tang et al. (2013)
Nested PCR	HBV, HCV, HEV	Increased sensitivity through multiple rounds	Increased risk of contamination	Hours	Chauhan et al. (2009)
Real-time PCR (qPCR)	HBV3, HCV, HEV	Quantitative measurement of target DNA/RNA	Requires expensive equipment and reagents	Hours	Vermehren et al. (2008)
Reverse Transcription PCR	HCV, HEV	Detects RNA viruses	Requires additional step for RNA-to-cDNA conversion	Hours	Vermehren et al. (2008)
Multiplex PCR	HBV, HCV, HEV	Simultaneous detection of multiple targets	Increased complexity and risk of nonspecific binding	Hours	Freeman et al. (1999), Tang et al. (2013), Shuber et al. (1995)
Digital-Droplet PCR	HBV, HCV, HEV	High precision and sensitivity, absolute quantification	Complex workflow, limited throughput, expensive reagents	Hours	Whale et al. (2012), Morisset et al. (2013), Mu et al. (2015)
Loop-mediated Isothermal Amplification (LAMP)	HBV, HCV, HEV	Rapid and simple process	Prone to nonspecific amplification, complex primer design	Minutes	Karami et al. (2011), Mori and Notomi (2009), Notomi et al. (2000), Freeman et al. (1999)

6.4.1 Real-Time Polymerase Chain Reaction

It was developed in the late 1980s to analyze RNA molecules quickly and amplify the sequence targets of those molecules. The reverse transcriptase enzyme converts the target RNA into single-stranded DNA (cDNA), which is then amplified using qPCR. An effective method for identifying RNA viruses such as hepatitis viruses (A, C, D, E) is RT-qPCR (Freeman et al. 1999; Tang et al. 2013; Cobo 2012). Real-time qPCR is a rapid technique used in clinical diagnostics as well as research that can identify viruses in blood and serum samples, including HEV, HCV, HDV, and HAV (Fig. 6.7). The sensitivity of this test varies depending on the sample, notwithstanding its speed (Freeman et al. 1999; Tang et al. 2013; Cobo 2012).

6.4.2 Nested PCR

This technique is an adaption of standard PCR that provides to increase the sensitivity and specificity. During PCR, this method utilizes the two sets of primers and DNA polymerase enzymes to amplify the target sequence in two steps. Nested PCR has a drawback: it takes longer to complete, increasing the chance of contamination. Despite this limitation, nested PCR has applications in science and medicine. It is particularly employed in the clinical identification of hepatitis viruses, in blood and serum samples. Moreover, the results of qPCR testing are often validated using nested PCR (Chauhan et al. 2009; Freeman et al. 1999; Tang et al. 2013; Nurtjahyani and Handajani 2013).

6.4.3 Multiplex PCR

It is an advanced PCR technique capable of simultaneously amplifying multiple target sequences using multiple primer sets. It is considered the most mature PCR technology and has shown success in detecting viral pathogens like HBV. Multiplex PCR is known for its speed, sensitivity, and cost-effectiveness, and it is widely used in both research and clinical settings, especially for genotyping. Additionally, it is an effective approach for identifying HBV and HCV genotypes in blood samples (Freeman et al. 1999; Tang et al. 2013; Shuber et al. 1995).

6.4.4 Loop-Mediated Isothermal Amplification

This technique is renowned for its exceptional precision, efficiency, speed, and specificity. It can produce results in less than 60 minutes, even under isothermal conditions. Notomi et al. first introduced loop-mediated isothermal amplification (LAMP) in 2000, and since then, it has gained widespread recognition and extensive utilization. RT-LAMP is a highly sensitive reaction that can produce detectable signals in just 5–10 minutes (Notomi et al. 2000). Commercial LAMP kits are now widely accessible, allowing their use in research and clinical settings (Karami et al. 2011; Mori and Notomi 2009; Notomi et al. 2000). The LAMP technique works in three distinct phases: an initial acyclic phase, an increasing cycling phase, and a final endurance phase. LAMP is considered an important significant diagnostic tool and is very useful in detecting viruses such as hepatitis B virus. The high speed, sensitivity, specificity, and versatility of this method make it suitable for research and diagnosis. Additionally, LAMP is recommended for epidemiological studies and treatment purposes (Freeman et al. 1999; Karami et al. 2011; Mori and Notomi 2009; Zhang et al. 2012).

6.4.5 *Droplet-Digital PCR*

To find mutational bias or the amount of target DNA material in a mixed cell population, the droplet-digital PCR, PCR (ddPCR) technique provides accurate and complete information. In some cases, ddPCR may be more useful and accurate than qPCR technology (Whale et al. 2012). The test sample was split into high-oil water and ddPCR technique before conventional PCR amplification. Thousands of PCR reactions occur simultaneously because each droplet is its own separate reaction. Droplets carrying the target are followed by a fluorescence signal in positive cases, and absent in negative cases. This method provides an estimate of the starting number of the sequence using Poisson statistical analysis (Morisset et al. 2013). The ddPCR-based technique shows potential for identifying single copies of HBV cccDNA. Templates derived from HBV DNA plasmids or HepG2. This is accomplished by using 215 HBV-infected cellular DNA extracts (Mu et al. 2015).

6.4.6 *Probe-Based Amplification Techniques*

There are three ways for identifying viral hepatitis: the cycling probe technique (CPT), the invader assay, and the ligase chain reaction (LCR) (Freeman et al. 1999). CPT is an isothermal signal amplification technology that detects trace quantities of DNA as a target sequence (Tang et al. 2013). The invader assay is a non-PCR approach for detecting DNA and RNA nucleic acids with great accuracy and sensitivity. It is used to score single nucleotide polymorphisms. It is also used to directly detect infectious pathogens such as hepatitis (A, B, and C) viruses (Germer et al. 2006). It is classified as a probe amplification approach in which two pairs of probes hybridize with neighboring single-stranded target DNA. LCR is quick, sensitive, and specific for simultaneous genotyping and mutant viral agents; nevertheless, its usage is limited and is employed for identifying resistant viral hepatitis agents in human serum samples (Wiedmann et al. 1994). Branched DNA (bDNA) method is a sandwich hybridization of nucleic acid technique used in amplifying signals molecular diagnostics to detect branched-structured target nucleic acids. It has been shown to be a sensitive, specific, and repeatable test for molecular viral diagnosis. The third generation of bDNA test commercial kit is now available for quantification of Hepatitis C RNA and HBV DNA (Ross et al. 2002). The hybrid capture assay (HCA) is a rapid viral molecular diagnostic technique that utilizes a denatured complementary DNA (cDNA) sequence as the target nucleic acid, a specific single-stranded RNA probe directly hybridized in a clinical specimen (Tang et al. 2006).

6.4.7 *Enzyme-Linked Immunosorbent Assay-Based Detection of Hepatitis Viruses*

Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used non-amplification methods for diagnosing viral infections, including hepatitis. It detects viral antigens or antibodies produced in response to an infection. This technique uses enzyme-conjugated antibodies to produce a measurable signal, typically a color change, in the presence of the target antigen or antibody (Fig. 6.7). ELISA is useful because of its simplicity, the effectiveness of costs, and high throughput, making it suitable for large-scale screening and initial diagnosis (Shuber et al. 1995). ELISA stands as a cornerstone in the diagnosis of viral hepatitis, offering a robust and versatile approach for detecting viral antigens and antibodies (Shuber et al. 1995). Employing the specific binding affinity between antigens and antibodies, ELISAs facilitate the identification of viral proteins or the host's immune response to the infection (Vermehren et al. 2008). This technique has been extensively utilized in the diagnosis of hepatitis virus infections as shown in Fig. 6.7. For instance, in HBV diagnosis, ELISA detects hepatitis B surface antigen (HBsAg), antibodies against HBsAg (anti-HBs), and antibodies against hepatitis B core antigen (anti-HBc), aiding in the differentiation of acute, chronic, and resolved infections (Weber et al. 1999). Similarly, ELISA is pivotal in detecting antibodies against HCV, indicating exposure to the virus, and antibodies against HAV, used for diagnosing acute HAV infections. ELISA's advantages include its high throughput, quantitative and qualitative data output, automation feasibility, and cost-effectiveness. However, challenges such as cross-reactivity and the window period post-infection should be considered (Shuber et al. 1995). Nonetheless, ELISA remains an indispensable tool in the clinical diagnosis and management of viral hepatitis.

6.4.8 *Next-Generation Sequencing (NGS)*

NGS allows for comprehensive analysis of viral genomes directly from clinical samples without prior amplification. Viruses like HCV, influenza, and HBV have substantial intra-host variability due to their high replication capacity and poor fidelity of the replication enzyme. During infection, mutations emerge spontaneously, leading to rapid adaptation and resistance to vaccines and antiviral drugs. Quasi-species, a mixture of closely related genomes within a host, allows viral populations to evolve resistance to vaccines and antiviral drugs (Woo and Reifman 2012). NGS provides detailed insights into viral genetic diversity, evolution, and resistance patterns. This method involves fragmenting the viral genome, sequencing the fragments, and then assembling the sequences to obtain the complete viral genome. Despite the numerous advantages offered by next-generation sequencing (NGS), its integration into clinical laboratories is impeded by several challenges.

These include turnaround time constraints, limitations in processing multiple samples per run, the high cost of sequencers, and the necessity for expertise in bioinformatics. These factors collectively hinder the widespread adoption of NGS in clinical settings, emphasizing the requirement for ongoing advancements and optimizations to fully harness its diagnostic potential (Dessilly et al. 2018; Jerome et al. 2019). NGS is extensively used to characterize intra-host variability in influenza, HCV, HIV, and HBV, primarily through ultradeep sequencing (UDS) of PCR-generated amplicons. HCV, due to its high replication rate and error-prone RNA polymerase, generates numerous mutations (Rong et al. 2010; Woo and Reifman 2012).

6.4.9 In Situ Hybridization (ISH)

ISH probes utilized to identify viral DNA or RNA in cells or tissues using nucleic acids. However, the next version of the ISH method uses the non-isotopic hapten digoxigenin, which gives better results. ISH is a rapid method for localizing hepatitis virus. Environmental parameters such as temperature, salinity, G + C content, and hybrid length all directly affect the safety of hybridization targets for sequencing and research (Cao et al. 2006; Tang et al. 2013). Peptide nucleic acid (PNA) is often used for fluorescent. Fluorescence in situ hybridization (FISH) is a quick and specific diagnosis technique for the detection of HBV. PNA FISH exhibits high sensitivity and specificity, enabled by probes that require efficient hybridization due to their strong affinity, rapid kinetics, and specificity for nucleic acids, including RNA (Cao et al. 2006; Pernagallo et al. 2012). It serves as an accurate and simple test for the detection of genomic DNA and RNA of viral hepatitis, including hepatitis A, B, and C, especially in certain regions. However, its usefulness is limited to a few comparisons. This pathobiological method has proven useful in detecting hepatocarcinoma in human hepatocytes. However, the main limitation of this complex hybridization technique is the easy access to the nucleic acid sequence in cells (Nuriya et al. 2010).

6.4.10 Biosensor-Based Detection Techniques

It utilizes biological molecules, such as antibodies or nucleic acid probes, immobilized on a sensor surface to detect viral antigens or genetic material. These biosensors convert the biological interaction into an electrical or optical signal, providing rapid and specific detection of viruses. Biosensors are highly sensitive and can be integrated into portable devices, making them suitable for point-of-care testing and real-time monitoring of infections (Singh et al. 2020). Ding et al. developed a label-free electrochemical biosensor for detecting oligonucleotides related to HBV sequences. The biosensor was hybridized with target DNA and used as an electrochemical indicator (Ding et al. 2008). Hashimoto et al. developed a microfabricated

disposable-type electrochemical DNA biosensor for HBV genomic DNA detection. The biosensor quantified HBV DNA in patients' sera, showing good correlation with competitive PCR, indicating its specific and quantitative detection capabilities (Hashimoto et al. 1998). Electrochemical biosensors detect biological processes such as enzyme reactions and nucleic acid hybridization (Uliana et al. 2008). Surface plasmon resonance (SPR) valuable instrument for scientific analysis, clinical diagnostics, the food industry, protecting the environment, and healthcare. It detects changes in refractive index when target molecules, such as HBV antigen, bind to nonspecific ligands on the surface (Scarano et al. 2010). Piezoelectric biosensors measure mechanical pressure using the piezoelectric effect, such as quartz crystal microbalance (QMC) for hepatitis B and C virus detection (Yao et al. 2008). The Apta sensor serves as a simple and specific biosensor for diagnosing a variety of clinical disorders. Enzyme-linked oligonucleotide assay (ELONA) and nanomechanical microcantilever methods are used to identify hepatitis viruses (Balogh et al. 2010). Several Apta sensors have been designed to detect hepatitis viruses, notably the very sensitive ELONA for HCV core antigen. This technique immobilizes an aptamer on a microtitration plate by adding an enzyme-conjugated monoclonal antibody after capturing the primary HCV antigen. Hwang and colleagues developed a nanomechanical microcantilever that uses RNA aptamers to efficiently detect HCV helicase also detect target the amount as minimal as 100 pg/ml (Hwang et al. 2007).

6.4.11 Microarray-Based Detection Technique for Viral Hepatitis

Molecular diagnostics continues to lead the way in microarray technology, sometimes referred to as lab-on-a-chip or biochip. By making the procedure smaller, it transforms diagnostics. Labeled RNA or DNA targets and immobilized nucleic acid probes hybridize in parallel on a solid surface according to the principle. The development of microarrays followed this step from Southern blots. By using scanning to identify probe-target hybridization, these techniques allow for the quick and precise investigation of a wide range of microbial genes, including portions of viruses (Ehrenreich 2006; Heller 2002; Lucchini et al. 2001; Skovgaard 2010; Tu et al. 2013; Wang et al. 2003). Microarray technologies are effective instruments for swiftly and precisely studying thousands of microbial genes, including viral sequences, by detecting hybridization between probes and labeled target sequences using a scanner. They are particularly useful for detecting various hepatitis viruses in research and clinical settings, enabling multi-sample diagnoses. However, they may not be ideal for detecting a small number of hepatitis agents in limited samples

(Cobo 2012; De Giorgi et al. 2019; Spiess et al. 2007; Xu et al. 2007). A study by Valeria De Giorgi and team developed a microarray-based pathogen chip to screen blood donors for transfusion-transmitted RNA viruses. The platform included multiple gene targets for each pathogen genome and balanced probe counts to 90–110. A workflow combining two applications was developed to increase target nucleic acid concentration. The RT-qPCR validation process involved quantifying positive specimens using the Altona RealStar RT-qPCR kit. The results showed that the pathogen chip correctly identified viral hepatitis (A, B, C), Chikungunya, dengue, HIV, HTLV I–II, West Nile, and Zika viruses between 10^5 and 10^2 copies/mL, and hepatitis E virus between 10^5 and 10^4 copies/mL (De Giorgi et al. 2019).

6.4.12 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Based Detection of Hepatitis Viruses

Zhang Feng's team utilized CRISPR RNA (crRNA) and Cas proteins, such as Cas13a, to develop a rapid detection method for lateral flow immunochromatography by integrating recombinase polymerase amplification (RPA) with CRISPR/Cas13a. This approach leverages biotin and FAM antigen for detection, offering a cost-effective, rapid, and highly specific diagnostic alternative (Abudayyeh et al. 2017; Freije et al. 2019; Kaminski et al. 2021). Nang Kham-Kjing and colleagues developed an RT-LAMP-coupled CRISPR-Cas12 assay for HCV RNA detection, which can be read using a lateral flow strip or fluorescence detector, demonstrating the versatility of CRISPR-Cas12 technology (Kham-Kjing et al. 2022). Similarly, the CRISPR-Cas13a system has been applied to detect hepatitis E virus (HEV) RNA with high sensitivity and specificity, offering a simpler alternative to traditional RT-qPCR methods, particularly in low-resource settings (Li et al. 2024) (Fig. 6.8).

CRISPR-Dx platforms utilize Cas proteins such as Cas12, Cas13, and Cas14, guided by RNAs, to detect and diagnose diseases. These systems exploit the collateral cleavage effect, enabling highly sensitive detection of genomic modifications associated with diseases like viral hepatitis, hepatocellular carcinoma (HCC), Zika, and Dengue virus (Gootenberg et al. 2017, 2018). The DNA endonuclease-targeted CRISPR trans reporter (DETECTR) system, for instance, uses Cas12a and single-stranded DNA fluorescent reporters for sensitive DNA detection, capable of differentiating viral subtypes like HPV in clinical samples (Chen et al. 2018). SHERLOCK, another CRISPR-based platform, employs Cas13 for RNA detection with single-nucleotide specificity and attomolar sensitivity, while CRISPR-chip enables targeted mutation detection without nucleic acid amplification (Gootenberg et al. 2017; Hajian et al. 2019).

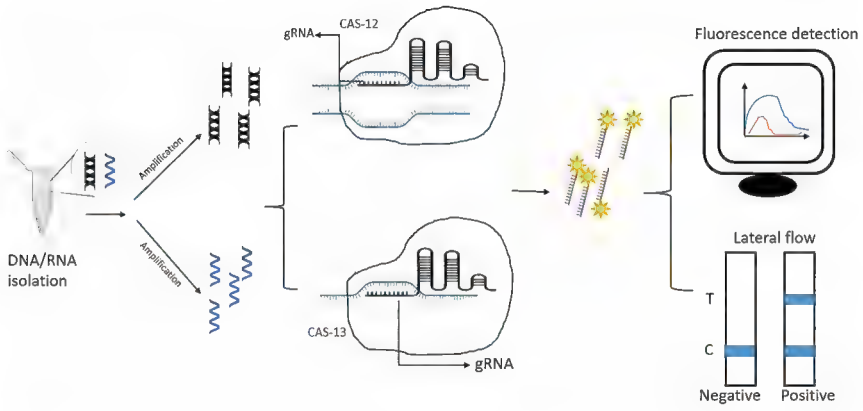

Fig. 6.8 CRISPR-based system for detecting viral hepatitis. DNA or RNA is extracted and directed using specific gRNAs. The patient's DNA is amplified using RPA, in contrast, RNA is amplified using RT-RPA followed by in vitro transcription. CRISPR-Cas12 or Cas13 enzymes bind to target sequences DNA and RNA, respectively, cleaving neighboring molecules and emitting fluorescent signals. Cas12 recognizes DNA viruses, while Cas13 targets RNA viruses. This rapid and sensitive detection utilizes CRISPR technology (Kham-Kjing et al. 2022; Wang et al. 2021; Tian et al. 2023)

6.4.13 Single-Pot LAMP-Mediated Engineered Brcas12b for Nucleic Acid Detection of Infectious Diseases (SPLENDID) Technique-Based Detection for Viral Hepatitis

Studies show that engineered BrCas12b variants (eBrCas12b) have increased activity and higher melting temperatures compared to AapCas12b and wild-type BrCas12b. The most thermostable eBrCas12b variant, when combined with RT-LAMP, eBrCas12b allows robust detection up to 67 °C, broadening the temperature range for CRISPR-mediated assays. This integration, known as SPLENDID, facilitates versatile primer design and is well suited for low-resource settings, offering sensitivity and specificity within 20 minutes (Nguyen et al. 2023). SPLENDID assay is being used for detecting HCV RNA in human serum samples. While achieving high specificity, accuracy, and sensitivity overall, it also identifies areas for improvement, particularly in detecting HCV genotypes other than GT1 (HCV genotype 1) (Hongjaisee et al. 2021). The findings suggest that optimizing LAMP primers and guide RNA specificity could enhance SPLENDID's performance across diverse HCV genotypes. Despite these limitations, the assay shows promise as a rapid and reliable diagnostic tool for HCV infection, with potential applications in clinical settings. While the SPLENDID test has several limitations, it is a promising quick point-of-care diagnostic tool. It requires careful primer design and additional optimization since it depends on the stability of the RT-LAMP reaction and the low sensitivity of the CRISPR-Cas trans-cleavage activity, for HCV patient samples,

especially for genotypes other than GT1 (HCV genotype 1), as its sensitivity was lower (Nguyen et al. 2023). Despite their advantages, non-amplification-based methods face challenges such as lower sensitivity compared to PCR-based techniques, potential cross-reactivity, and the need for specialized equipment and trained personnel. Future developments aim to enhance the sensitivity and specificity of these methods and make them more accessible and cost-effective for widespread clinical use.

6.5 Challenges

Molecular testing for hepatitis viruses is complicated by the different types of hepatitis viruses, including HBV and HCV. Response to hepatitis testing at this time, people with high incomes face challenges such as lack of awareness, limited resources, access to reliable diagnostics, limited laboratory capacity, weak monitoring systems, high levels of stigma, social exclusion and lack of testing guidelines, and lack of planning and policy and funding. RNA NAT (nucleic acid testing) like RT-PCR, qRT-PCR, LAMP, branched DNA (bDNA) assay, digital PCR (dPCR), and CRISPR-based detection systems (e.g., SPLENDID, SHERLOCK, DETECTR) are recommended as the preferred testing strategy for viremic infection, while core HCV antigen detection may be considered. HBV DNA NAT after reactive HBsAg serology was recommended for treatment decisions and response monitoring according to WHO guidelines on hepatitis B management. There are different methods for the detection of viral hepatitis which have some advantage and limitations in time, sensitivity, specificity, and practical application. ELISA is a low-cost, high-sensitivity, and specific protein or peptide-based assay that needs advance preparation. The method's high sensitivity enables the detection of viral antigen concentrations of less than 1 ng per milliliter in specimens acquired directly from patients. In situ hybridization (ISH) for the detection of viral RNA within tissue at subcellular resolution allowing spatial typing with high specificity. PCR techniques for discovering and measuring viral genomes. Because of its speed, sensitivity, and adaptability, this technique has become an important element of the diagnostic laboratory's normal work, and numerous commercial test systems are available. Another technique is quantitative real-time polymerase chain reaction (qRT-PCR), which provides viral load information but it is expensive and requires special instrumentation, in addition to fluorophore that can bind to the PCR products. Commercial microarrays are available for the simultaneous detection wide range of respiratory viruses by integrating the findings of multiplex PCR and RT-PCR operations with a solid chip or liquid bead area containing oligonucleotides for different viruses. Such methods have been developed to simultaneously detect and sequence human papillomavirus in clinical practice. Transferring the technology to the routine diagnostic laboratory remains a challenge, primarily due to the need for faster times, higher speeds, reliable diagnostic techniques, and a variety of treatment options. But the use of microarray technology is likely to lead to another revolution in diagnostic

laboratories. Next-generation sequencing is used in viruses to identify unknown or documented viruses in infectious diseases, diagnosis of difficult-to-treat diseases using autopsy archives, screening of infected vaccines, analysis of virus species in clinical samples, and study of differences and evolution of viruses. Specific genes and the study of the human genome in health and disease. However, full use of modern virus detection technologies has not yet been realized and is likely to complement rather than replace existing methods. Falling costs have pushed the technology from large companies into larger areas. However, due to the large amount of data, there are still development problems, including the calculation and calculation of the data needed. The CRISPR12a/Cas13a detection method is inexpensive and does not require expensive equipment. A standard SHERLOCK test costs is lower than the competing real-time PCR method. It is evolving very quickly and changing the way viruses are detected, using the same existing methods to meet different needs. Transporting blood samples to a testing center from a remote site typically necessitates additional logistical support in low-resource settings or countries. The dried blood spot testing (DBS) method of sample collection can be used to solve this problem (Kania et al. 2013; Komada et al. 2015; Vinikoor et al. 2015a, b). Nucleic acid testing for HBV screening is prevalent in industrialized countries but less in low-income ones due to cost and skilled labor requirements.

6.6 Conclusion

Viral hepatitis detection uses non-amplification-based molecular diagnostics, PCR-based techniques, and CRISPR-based procedures, offering accurate and sensitive detection options like ELISA, hybridization assays, microarray, biosensor, and next-generation sequencing. From these techniques, we can directly identify the viral nucleic acids, resulting in rapid and specific results. Nucleic acid-based methods are useful for rapid, accurate identification and viral detection with high sensitivity and specificity. Advances in virological testing methods have replaced conventional clinical testing methods, such as those used in research settings. PCR-based technologies, including conventional, real-time, and structured PCR, are highly sensitive and specific and provide accurate identification. Nucleic acid-based methods in the virology laboratory are useful for rapid, accurate identification and diagnosis of viral agents with sensitivity and specificity. Research-based studies identify viruses using transcription factors, resulting in specificity, specificity, and specificity. Enzyme-linked immunosorbent assay (ELISA) is an inexpensive and widely available test with the advantages of simplicity, rapid results, and high throughput. Amplification techniques are superior in sensitivity and early detection but are expensive and sophisticated, whereas non-amplification approaches are more accessible and faster but may compromise some sensitivity and specificity. Further study and advancement in this field is needed in order to enhance the sensitivity, specificity, and accessibility of viral hepatitis detection methods, resulting in better patient and public health outcomes.

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Chapter 7
Introduction to Molecular Diagnosis of Meningitis

Vipin Kumar Sharma, Shilpa Raina, Shrishti Lakhera, and Jyoti Sangwan

Abstract Meningitis, an inflammation of the meninges continues to be a critical global issue with significant morbidity and mortality, especially in vulnerable populations such as infants, the elderly, and immunocompromised individuals. Meningitis is considered to be a significant public health concern. The chapter provides insight into current literature on meningitis, majorly focusing on its diagnosis which plays a significant role in the management of this fatal disease. Due to various limitations in conventional methods of diagnosis, advances in molecular diagnostics have begun to address those problems, offering a scope to combat the serious consequences offered by this disease. The future of next-generation sequencing in meningitis diagnosis is promising. However, challenges persist, particularly in areas with limited healthcare access. Continued public health efforts and research are crucial to reduce the global burden of meningitis and to improve patient prognosis. This chapter aims at understanding the conventional diagnosis approaches for meningitis with the limitations and emergence of molecular methods for a better diagnosis, as well as roadblocks in their implementation.

Keywords Meningitis · Molecular-diagnosis · PCR · Multiplex PCR

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7.1 Introduction

Meningitis is a lethal condition characterized by inflammation of fluids and membranes that surround the brain and spinal cord. It is an infection in the subarachnoid space followed by an inflammatory process in the central nervous system (CNS). The condition can result in memory problems, light sensitivity, hearing loss, seizures, coma, rise in intracranial pressure, stroke, or subsequent death. The meninges are affected by this condition. Meninges comprises dura matter, arachnoid matter, and pia matter membranes which line up with the skull enclosing the brain as well as the spinal cord (Fig. 7.1).

Meningitis could be a result of infectious or non-infectious processes. The etiologic agents in infectious meningitis include groups of bacteria, fungi, parasites, and viruses. Each type requires a special prognosis and therapeutic approach. Early recognition is crucial to avoid severe complications. Various types of meningitis are described below:

7.1.1 Bacterial Meningitis

It has been considered a cause of concern, a potentially life-threatening condition. The symptoms include high fever, headache, stiff neck, photophobia, nausea, vomiting, and altered mental issues (Diallo et al. 2021). The transmission of this condition occurs through close contact, childbirth, and through droplets. To diagnose bacterial meningitis, Lumbar puncture of Cerebrospinal fluid (CSF) is obtained followed by gram staining and polymerase chain reaction (PCR). Major bacteria

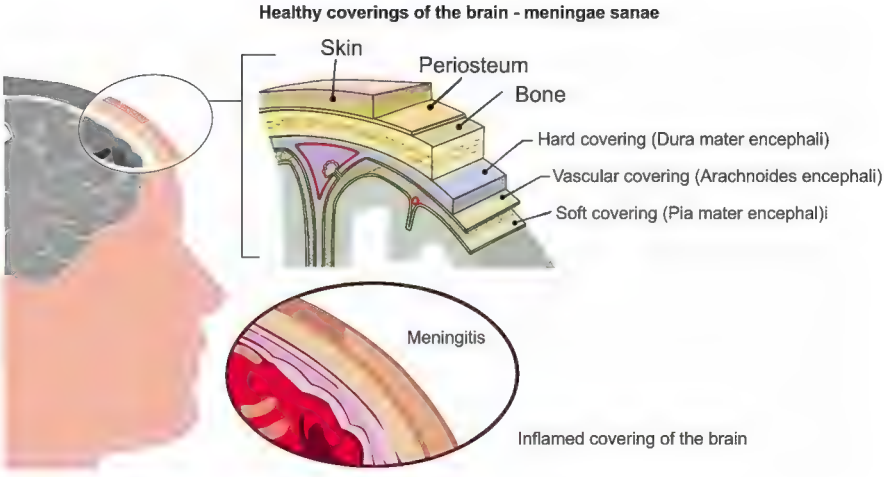

Fig. 7.1 Illustrates meninges comprising its major layers affected in meningitis condition

responsible for this condition include *S. pneumonia, Group B streptococcus, H. influenza, and N meningitidis*. As the disease is diagnosed, treatment is required with broad-spectrum antibiotics. Since the disease is associated with inflammation, corticosteroids are given to the patient (Albuquerque et al. 2019; Roos 2000; Van De Beek et al. 2012).

7.1.2 Viral Meningitis (Aseptic Meningitis)

Also known as Aseptic meningitis, it is generally a less severe form of meningitis. Viral pathogens include non-polio enteroviruses, mumps, herpes viruses, etc. The symptoms are similar to bacterial meningitis, just less severe like fever, headache, neck stiffness, fatigue, diarrhea, etc. The CSF as obtained from viral meningitis patients shows elevated levels of WBCs, normal glucose levels, and a negative bacterial culture indication. To treat these conditions, antiviral medications specific to viral infection are provided to combat the inflammation (Kohil et al. 2021).

7.1.3 Fungal Meningitis

It is a rare form of meningitis, typically affecting people with a compromised immune system such as HIV/AIDS, cancer, or people undergoing organ transplants. Fungal meningitis is associated with host immunity when it is compromised in conditions like HIV/AIDS, or cancer. The symptoms are similar to viral and bacterial meningitis. The transmission typically occurs via inhalation of fungal spores from the surroundings. CSF analysis shows an increase in WBC, low glucose levels, and a positive fungal culture test. Antifungal therapy is provided along with drugs Amphotericin B, Flucytosine, and Fluconazole (Gottfredsson and Perfect 2000).

7.1.4 Parasitic Meningitis

Parasitic meningitis is rare and caused by parasites like *Naegleria fowleri, Angiostrongylus cantonensis,* and *Toxoplasma gondii*. The transmission is via ingesting contaminated food and water. *Naegleria fowleri* infection is fatal and requires immediate administration of miltefosine. The prognosis for parasitic meningitis is poor, especially when amoebic infections come into play (Visvesvara et al. 2007).

7.1.5 *Non-infectious Meningitis*

This form of meningitis occurs without any detectable infection via pathogens. Non-infectious meningitis can occur when the immune system is compromised. This can happen due to autoimmune disorders like lupus, certain medications like NSAIDs, cancer, or inflammatory diseases like sarcoidosis. The symptoms of this are similar to those of other forms of meningitis. However, the CSF examination typically does not show signs of infection. Additional tests are required to confirm the underlying cause. Since non-infectious meningitis is often related to autoimmune conditions, treatment usually involves corticosteroids or immunosuppressants (Dubé et al. 1992).

Meningitis may be acute, but it sometimes gets chronic, the latter is characterized by symptoms that stay up to several weeks or months, with gradual onset. Tuberculosis, fungal infections, malignancies, and autoimmune diseases are a few of the underlying causes of this condition. The condition comes with various symptoms including headache, fever, nausea, and neurological symptoms (Cranial nerve palsies). It is differentiated from other forms of meningitis as long-term neurological damage can occur if treatment is not provided urgently (Thakur and Wilson 2018).

7.2 Conventional Approaches for Diagnosis of Meningitis

The conventional method for diagnosis of meningitis relies on clinical assessment, cerebrospinal fluid (CSF) analysis, and microbiological techniques. While these methods have been utilized for decades, they have limitations in terms of their sensitivity, specificity, and time required for results. The causative micro-organism and appropriate therapy are decided once the CSF Gram staining examination is done. Although CSF examination is considered to be a top-notch test for bacterial meningitis diagnosis. However, its accuracy can be compromised if the patient is already under antimicrobial drugs. Molecular diagnostic approaches aim to address these limitations by offering more precise and rapid identification of pathogens. Figure 7.2 illustrated the overview of various diagnostic approaches for meningitis.

7.2.1 *Microbial Culture*

By this method, bacteria like *S. Pneumonia* (Gram-positive), *Streptococcus agalactiae* (Gram-positive), *Haemophillius Influenza (*Gram-negative), *Escherichia Coli (*Gram-negative) can be identified by forming their colonies and further identification. The first and foremost step for diagnosing the condition is clinical assessment. Initial diagnosis is evaluating symptoms like fever, headache, neck stiffness, mental abnormality, and photophobia. The individual is physically examined for Brudzinski's and Kernig's signs to detect meningeal irritation. Different diagnostic methods are summarized below:

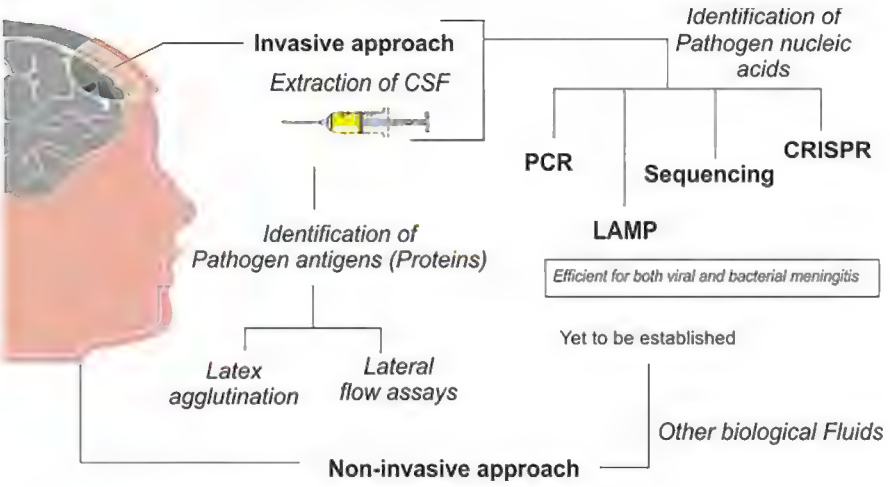

Fig. 7.2 Summary of the diagnosis of meningitis

7.2.2 CSF Analysis

This method includes lumbar puncture of CSF, followed by cell counting, checking levels of glucose and protein level, and lastly confirmation via gram staining and culture in microbiology laboratories. The gold standard of diagnosing meningitis, where lumbar puncture is performed to collect cerebrospinal fluid is often followed either by some conventional approaches or occasionally molecular diagnosis. Below are some common conventional approaches often followed post-CSF extraction.

- Cell count—elevated levels in CSF indicate inflammation. Polymorphonuclear leukocytes suggest bacterial meningitis whereas lymphocytes suggest viral or tuberculous meningitis in the patient (Young and Thomas 2018).
- Glucose and protein estimation—both glucose and protein levels are indicative of the type of meningitis. Low glucose level along with elevated protein levels show the presence of bacterial infection while viral meningitis presents normal glucose levels and moderately increased levels of protein. Viral infections can be diagnosed by combining CSF cell cultures and identification of specific antibodies (IgG and IgM) in serum or CSF.
- The Gram staining method is an additional rapid and inexpensive process that provides etiology of the pathogen in fungal and bacterial meningitis. The procedure involves the use of enriched media (blood or chocolate agar) where colonies are formed and the pathogen is identified within 24–48 h. However, it comes with some limitations such as a longer wait period until results are obtained and the inability of some pathogens to be cultured on media (Putz et al. 2013), limiting the use and reliability.

- Ziehl-Neelsen Staining is utilized in tuberculosis meningitis and helps in detecting acid-fast bacilli like *Myobacterium tuberculosis*. The sensitivity of this test is low, whereas if the bacterial load is low the acid bacilli might be missed.

7.2.3 Imaging Methods

Imaging methods used for the diagnosis of meningitis primarily include *computed tomography (CT) scans* and *magnetic resonance imaging (MRI)*. CT scans are often performed first to rule out complications such as brain swelling, hydrocephalus, or abscesses before a lumbar puncture. However, MRI is more sensitive in detecting meningeal inflammation, showing findings like leptomeningeal enhancement with contrast, ventriculitis, and cerebral edema. Diffusion-weighted imaging (DWI) in MRI can also help differentiate bacterial meningitis from viral or fungal causes. Though imaging supports diagnosis, cerebrospinal fluid (CSF) analysis via lumbar puncture remains the gold standard for confirming meningitis. Imaging methods used for the diagnosis of meningitis primarily include *computed tomography (CT) scans* and *magnetic resonance imaging (MRI)*. CT scans are often performed first to rule out complications such as brain swelling, hydrocephalus, or abscesses before a lumbar puncture. However, MRI is more sensitive in detecting meningeal inflammation, showing findings like leptomeningeal enhancement with contrast, ventriculitis, and cerebral edema. Diffusion-weighted imaging (DWI) in MRI can also help differentiate bacterial meningitis from viral or fungal causes. Though imaging supports diagnosis, cerebrospinal fluid (CSF) analysis via lumbar puncture remains the gold standard for confirming meningitis. Although a sensitive enough and clinically preferred method, it remains an expensive choice for the patients and molecular methods can still provide relief to patients, provided they attain a similar level of sensitivity and specificity.

7.2.4 Latex Particle Agglutination Tests

The Latex Particle Agglutination Test (LPAT) is a rapid diagnostic test used to detect bacterial antigens in cerebrospinal fluid (CSF) for the diagnosis of bacterial meningitis. It relies on latex beads coated with specific antibodies that agglutinate in the presence of bacterial antigens, forming visible clumps. The test can detect major causative pathogens, including Streptococcus pneumoniae, Neisseria meningitidis (serotypes A, C, Y/W135)), *Haemophilus influenzae* type b, Escherichia coli K1, and Group B Streptococcus. The procedure involves mixing CSF with latex reagent on a slide and observing for agglutination within minutes. LPAT is highly sensitive and faster than culture methods, making it useful in urgent cases. However, it has limitations such as false positives, lower specificity compared to PCR, and reduced sensitivity if bacterial loads are low or if antibiotics were administered

before testing (Roos 2000). Recent advancements focus on improving sensitivity and specificity through better antigen detection and combining LPAT with molecular diagnostic techniques.

7.2.5 *Counter-Immuno-Electrophoresis*

Counter Immunoelectrophoresis (CIE) is a rapid serological test used for detecting bacterial antigens in cerebrospinal fluid (CSF) for the diagnosis of bacterial meningitis. It is based on the principle of electrophoresis, where bacterial antigens and their specific antibodies migrate towards each other in an agar medium under an electric field, forming a visible precipitation line if the antigen is present. CIE can detect pathogens such as Streptococcus pneumoniae, Neisseria meningitidis, *Haemophilus influenzae* Type B, *Escherichia coli K1*, and Group B Streptococcus. This method is faster than culture techniques and more sensitive than conventional agglutination tests. However, its limitations include the requirement for specialized equipment, skilled personnel, and decreased sensitivity in cases with low bacterial antigen concentrations. Recent advancements focus on enhancing antigen detection sensitivity and integrating CIE with molecular methods for improved accuracy in diagnosing bacterial meningitis.

7.3 Epidemiology of Meningitis and Need for Molecular Diagnosis

The worldwide incidence rates of this purulent condition have been estimated to be 20 cases per 100,000 people and it varies as per geographical regions. Most of the outbreaks have been detected in the Sub-Saharan region. Due to such a burden, the region is also termed an African meningitis belt. Iran is also affected by this condition where the incidence rate reached 2.7 per 100,000 people in 2008. The main reasons could be a lack of sufficient resources, the high cost of health care, lack of knowledge regarding routine vaccination, improper management of public health issues, and commercial diagnostic methods (Moradi et al. 2021). World Health Organization has stated meningitis as a medical emergency and concluded that in the population of affected individuals, one in five people have severe complications and 1 in every 6 people dies due to its effects. In 2015, there were reports of 8.7 million cases of meningitis worldwide along with 379,000 fatality rates due to this condition. People residing in close proximity like overcrowded places, refugee camps, or mass gathering settings have a high risk of getting meningitis. Immune deficiencies (like HIV), and active passive smoking could also raise the risk of this condition. Children younger than 5 years of age and adults who are older than age of 65 years (Moradi et al. 2021).

Meningitis is a medical emergency and needs to be diagnosed as soon as possible to avoid its serious consequences. The first step in the diagnosis procedure is the identification of potential pathogens causing the condition. Lumbar puncture of CSF (Cerebrospinal fluid) is accomplished to detect the disease. Further confirmation is done via rapid diagnostic tests like PCR, LAMP, and multiplex assay (Diallo et al. 2021). The conventional methods provide results with great accuracy but are time-consuming and not each type of human pathogen can be cultured. Meningitis being a chronic condition needs adequate therapy urgently to avoid high mortality rates. However, conventional methods are still in use and they remain essential especially where advanced molecular techniques are limited.

7.4 NAAT-Based Approaches for Meningitis

Molecular methods like polymerase chain reaction is a sensitive method which detects pathogens more specifically and rapidly. It can be exploited to detect pathogens in CSF samples where the culture results are found to be negative or the patients undergoing any antimicrobial therapy. Moreover, this method utilizes a small volume of clinical samples and provides more accurate results as compared to conventional methods. Previously, conventional PCR was used for the detection, where the pathogen DNA is amplified using special primers specific to the pathogen DNA sequence. The results are analyzed further to classify it as a bacterial, viral, or fungal infection. This method can detect pathogens such as *Neisseria meningitidis*, *Streptococcus pneumonia*.

Various other versions of the PCR as well as broader umbrella group nucleic acid amplification techniques (NAAT) include real-time PCR (RT-PCR), qualitative and quantitative PCR (qPCR), and loop-mediated isothermal amplification assays (LAMP). These techniques help overcome the limitations of the culture-based method by providing faster and more accurate identifications of the causative agents. Advancements in PCR techniques have transformed the diagnosis of meningitis by providing faster, more accurate and comprehensive detection of pathogens. PCR involves the amplification of pathogen DNA as obtained from CSF samples of patients.

7.5 Quantitative Real-Time PCR (qRT-PCR) and Reverse Transcription PCR (RT-PCR)

For rapid diagnosis of meningitis, these PCR techniques are highly useful. These methods enable the quick detection and quantification of pathogens in a single run. By measuring the expression levels of various pathogens, qRT-PCR provides immediate information on the pathogen load, aiding in prompt and accurate diagnosis.

Table 7.1 Summary of popular commercially available molecular diagnostic kits for viral meningitis using PCR

Kit name[a]	Manufacturer	Target pathogens	Sample type	Key features
VIASURE Viral Meningitis Panel Real-Time PCR Detection Kit	Certest Biotec	Detects DNA and RNA from adenovirus, cytomegalovirus, Epstein-Barr virus, and other viral meningitis pathogens	CSF	Real-time PCR for qualitative detection of multiple viruses associated with meningitis
Bosphore Neonatal Meningitis Panel Kit	Anatolia Geneworks	Detects Escherichia coli (*uidA* gene), *Streptococcus agalactiae* (*cAMP* gene), and *Listeria monocytogenes* (*hly* gene)	CSF, plasma, serum	Real-time PCR-based detection of bacterial pathogens that can cause neonatal meningitis
AccuPower Neisseria meningitidis Real-Time PCR Kit	Bioneer	Detects *Neisseria meningitidis*, a bacterial cause of meningitis	CSF	Real-time PCR for specific detection of *N. meningitidis* with high sensitivity and specificity

[a]Order/sequence does not signify the preference of kits or their merit. Editors or Authors do not endorse any of the kits

RT-PCR is a critical technique for diagnosing viral meningitis. It starts with the conversion of RNA into complementary DNA (cDNA) using specific primers. The cDNA is then amplified to detect the presence of RNA viruses. RT-PCR is especially valuable for identifying enteroviruses in cases where traditional methods may fail, providing accurate and timely detection of viral pathogens (Table 7.1).

7.6 Multiplex PCR

Unlike the PCR method which targets one pathogen at a time, now multiplex PCR are being utilized in laboratories to simultaneously target multiple pathogens in a single run with minimal clinical sample. The diagnostic system becomes more efficient through reduced testing time and enhanced diagnostic precision from this technique. The utilization of multiplex PCR brings exceptional benefits to cases that evade traditional diagnosis methods including patients given antimicrobial treatment or individuals presenting with negative culture CSF results.

The testing methods of molecular diagnostics with multiplex PCR demonstrate better benefits than traditional diagnostic approaches do. Multiplex PCR surpasses Gram staining and latex agglutination tests because it detects even minimal bacterial, viral, or fungal DNAs with high precision regardless of sample quality or pathogen levels. A major procedural advantage of CSF meningitis diagnosis using small samples derives from avoiding patient distress linked to lumbar puncture as well as its associated negative effects (Sundelin et al. 2023).

The multiplex PCR diagnostic process starts with clinical specimen collection, followed by bacterial culture, CSF sample preparation, and bacterial DNA extraction. It includes clinical bacterial lysate preparation followed by primer selection, reaction condition optimization, and specificity testing before conducting a diagnostic test performance assessment. The targeted pathogens undergo specific amplification through multiplex-PCR diagnosis which provides both fast and precise detection methods. Patient management gets better due to quick and efficient testing which allows early prescription of suitable antimicrobial medications (Sharma et al. 2022).

Various prototype assays have been developed targeting multiple pathogens. Recently, a TaqMan array has been developed which was tested in specimens from West Africa which indicated diverse infectious micro-organisms in the sample. The multiplex test helps in the identification of each type of pathogen whether it is a bacterial, fungal, or viral infection. The first ME panel to be commercialized and approved by the FDA was Filmarray Meningitis/Encephalitis ME (BioFire Diagnostics, UT) This panel can detect 6 bacteria, and 7 viral pathogens along with 1 fungal pathogen. Multiplex test provides rapid results which not only help in optimal patient management but also reduce usage of unnecessary antibiotic consumption (Table 7.2). The main advantage of this test is that it encompasses the limitations of conventional methods by working with patients undergoing antimicrobial therapies. Along with many advantages ME panels also come with certain limitations as this kit/panel is specific to certain specific micro-organisms as included in the panel. It fails to identify other causative pathogens. The assays result in false positive and false negative results due to contamination or low quantity of pathogens (Albuquerque et al. 2019; Vila et al. 2021). Multiplex PCR panels have revolutionized the diagnosis of meningitis by enabling rapid and simultaneous detection of multiple pathogens.

7.6.1 Loop-Mediated Isothermal Amplification Assays

The LAMP assay utilizes a single DNA target and amplifies it in isothermal conditions. DNA polymerase extends the target DNA strand without needing any temperature alternation. So, with basic equipment and normal conditions, this assay can be performed. LAMP assays have been utilized for the detection of *N. meningitidis*, *S. pneumonia,* and *H. influenza*. LAMP is much simpler, yet faster as compared to PCR. Such assays are highly required in hospital setups which don't have access to expensive equipment and have limited resources (Vila et al. 2021).

7.6.2 Next Generation Sequencing (NGS)

NGS has emerged as a powerful tool in infectious disease diagnosis, more particularly in complex cases like meningitis where the identification of the pathogen involved is challenging. It offers a more comprehensive approach as compared to

Table 7.2 Summary of current multiplex PCR panels used for meningitis diagnosis, highlighting their targeted organisms

Kit name[a]	Manufacturer	Target pathogens	Sample type	Key features
BioFire FilmArray Meningitis/Encephalitis Panel (Obaro et al. 2023; López et al. 2024)	BioMérieux	Bacteria: *Escherichia coli* K1- *Haemophilus influenzae- Listeria monocytogenes- Neisseria meningitidis- Streptococcus agalactiae- Streptococcus pneumoniae* Viruses: Cytomegalovirus (CMV)- Enterovirus (EV)- Herpes simplex virus 1 (HSV-1)- Herpes simplex virus 2 (HSV-2)- Human herpesvirus 6 (HHV-6)- Human parechovirus (HPeV)- Varicella zoster virus (VZV) Yeast:- *Cryptococcus* (*C. neoformans*/*C. gattii*)	CSF	Multiplex PCR panel for rapid detection in ~1 h. Fully automated sample-to-answer system. High sensitivity (94.2%) and specificity (99.8%)
QIAstat-Dx Meningitis/Encephalitis (ME) Panel (Cuesta et al. 2024; Sharma et al. 2022)	QIAGEN	Bacteria: *Escherichia coli* K1- *Haemophilus influenzae- Listeria monocytogenes- Neisseria meningitidis- Streptococcus agalactiae- Streptococcus pneumoniae- Mycoplasma pneumoniae- Streptococcus pyogenes*Viruses:- Herpes simplex virus 1 (HSV-1)- Herpes simplex virus 2 (HSV-2)- Human herpesvirus 6 (HHV-6)- Enterovirus- Human parechovirus (HPeV)- Varicella zoster virus (VZV)Fungus:- *Cryptococcus neoformans/gattii*	CSF	Provides results in approximately 79 min. Displays amplification curves and cycle threshold (Ct) values for detected pathogens
Multiplex PCR Detection for Meningitis/Encephalitis (MME-18) (Si et al. 2022)	Research-based	Bacteria: *Neisseria meningitidis- Mycobacterium tuberculosis- Haemophilus influenzae- Mycoplasma pneumoniae- Streptococcus pneumoniae- Acinetobacter baumannii- Streptococcus agalactiae- Escherichia coli* K1- *Listeria monocytogenes* Fungus:- *Cryptococcus neoformans* Viruses:- Enterovirus (EV)- Mumps virus (MuV)- Herpes simplex virus 1 (HSV-1)- Herpes simplex virus 2 (HSV-2)- Varicella zoster virus (VZV)- Epstein-Barr virus (EBV)- Cytomegalovirus (CMV)- Human herpesvirus 6 (HHV-6)	CSF	Designed to identify 18 common pathogens in CSF. Aims to provide comprehensive coverage for meningitis/encephalitis pathogens

[a]Order/sequence does not signify the preference of kits or their merit. Editors or Authors do not endorse any of the kits

traditional methods and allows the identification of a wide range of pathogens at the same time.

- Whole genome sequencing—it involves sequencing the entire genome of the infectious agent. The sequence hence obtained provides a comprehensive detail about the pathogen like its genetic makeup and antimicrobial activity.
- Metagenomics—allows genome sequencing of pathogens, without any prior knowledge. It helps in the identification of novel infectious agents. This method is particularly useful when culture-negative meningitis, in situations where traditional methods fail.
- Targeted NGS—it focuses on targeting specific regions of the pathogen genome. For example, the 16 s RNA gene is targeted for bacterial identification whereas in the case of fungi, the ITS region is identified.

NGS provide unbiased metagenomic sequencing without prior suspicion of the pathogen. It helps in keeping track of newly emerging strains of pathogen or resistance patterns. All this information is crucial for guiding vaccine development and in turn, helps in studying the epidemiology of outbreaks and evolution of pathogens.

Microarray Analysis—microarrays allow simultaneous analysis of thousands of sequences, making them crucial for pathogen detection. They utilize probes that are attached to solid surfaces onto which a complementary sequence of pathogens hybridizes. The identification of pathogens is done by analyzing the hybridization pattern. This method is utilized in case of polymicrobial infections.

7.7 Challenges in Molecular Diagnosis of Meningitis

Irrespective of the significant advantages offered by molecular methods, they still face several challenges that limit their usage and effectiveness. The complexity of sample preparation is a tedious process which requires special types of equipment and technical expertise. This offers a disadvantage to the people residing in limited-resource regions where the burden of meningitis is the highest. Molecular technologies like NGS and PCR require careful handling to avoid contamination which could result in false positive results, hence manipulating the findings.

The routine use of molecular diagnosis particularly NGS, is difficult as it requires high cost and requirement of bioinformatics expertise to analyze and interpret complex data.

Multi-resistance of microorganisms against antibiotics is a major problem. Their re-emergence in mutated form fails current vaccines for the management of the disease. Antimicrobial resistance has emerged as an urgent issue creating global health challenges. It is considered to be causing a daunting threat to the future healthcare system. Antibiotic resistance is the ability of microorganisms to develop resistance against antibiotics that were previously thought to be effective against them. The microbe doesn't get affected in the presence of the antibiotic and continues to reproduce.

There is a high need for a test that detects each pathogen and can identify the antimicrobial resistance for the same. It should be inexpensive and easy to perform to reduce time in the diagnosis process, hence leading to faster disease management. Such tests should reach a population where lack of knowledge is a major cause of its widespread. Free vaccinations are a must to eradicate this condition.

One of the major challenges with meningitis is the development of vector-borne diseases. They can spread globally hence becoming more lethal and a global concern (Vila et al. 2021). The molecular diagnosis of meningitis has significantly advanced over the past decade, but there are still some areas where further research and development is required. In-depth knowledge of the type of meningitis, pathogen adapt its evolution and replication, natural history of infection, virulence factors, and target site which are necessary to fight against this catastrophic infection.

7.8 Future Prospects in Molecular Diagnosis of Meningitis: Non-invasive Approaches and Biomarker Innovations

Meningitis, a life-threatening inflammation of the meninges, demands rapid and precise diagnosis to guide therapy, particularly given the divergent treatments for bacterial and viral etiologies. Traditionally, CSF analysis via lumbar puncture has been the gold standard for identifying CNS infections, but this comes with pain, bleeding, risk of infection, and in some cases brain herniation in patients having high intracranial pressure. These challenges have led researchers to explore non-invasive, rapid molecular techniques and biomarker-based strategies to revolutionize meningitis diagnosis and management.

The invasiveness and risks associated with CSF sampling underscore the urgent need for alternatives. Recent studies have focused on highly sensitive and specific nucleic acid amplification techniques, biomarker identification, and metagenomic sequencing to enable rapid pathogen detection from alternate sample sources like blood, saliva, and urine. Such approaches aim to reduce procedural complications while accelerating diagnosis, critical for improving outcomes in acute settings (Poplin et al. 2020).

While PCR-based techniques have gained attention and tend to have improved diagnostic accuracy, their reliance on pathogen-specific primers limits utility for novel or unknown agents. NGS overcomes this by analyzing the entire genomic content in the sample, proving invaluable in identifying elusive pathogens in meningitis and encephalitis cases. Multiplex PCR also emerges as an important diagnostic tool that identifies pathogens when bacterial cultures provide no results due to antibiotic treatment or slow-growing bacterial conditions. These multiplex PCR panels enhance the diagnostic process by providing rapid, comprehensive, enabling simultaneous detection of multiple pathogens from minimal CSF volumes. These technologies expedite targeted treatment, directly impacting survival rates (Wilson et al. 2019).

CSF biomarkers have emerged as pivotal tools for differentiating bacterial meningitis (BM) from aseptic meningitis (AM). Cytokines like TNF-α, IL-1β, IL-6, and IL-8 exhibit marked elevations in BM, with IL-6 correlating strongly with neurologic complications. The assessment of these cytokines establishes their ability to diagnose between BM and AM ranging from 46.5% to 93% in sensitivities and 81% to 100% in specificities. The acute-phase proteins C-reactive protein (CRP), α1-antitrypsin (AAT), and α1-acid glycoprotein (AAG) and haptoglobin (HPT), ceruloplasmin (CER) demonstrate a broad range of elevations in BM patients which particularly makes CRP effective because of its IL-6-driven inflammatory response. The diagnostic sensitivity of CSF CRP ranges from 70% to 100% while its diagnostic specificity reaches from 90% to 95%. Acute-phase proteins AAT, AAG, HPT, and CER have significant elevations in BM which leads to diagnostic sensitivities of 50–83% and diagnostic specificities above 90% to 97%. BM produces higher levels of the essential anti-inflammatory mediator cortisol compared to both AM and control groups and achieves a diagnosis of BM with 82% accuracy together with 100% specificity. Novel markers like sTREM-1, HMGB1, and oxidative stress indicators (nitrite, 8-OHdG) provide additional diagnostic and prognostic insights. For instance, sustained 14-3-3 protein levels predict poor outcomes while declining TNF-α and nitrite levels signal therapeutic success. Additionally, CSF peptide fragments associated with inflammation include complement components C3, C4-A, C4-B, C1q subcomponent B chain, C5, and C9, as well as haptoglobin, C-reactive protein, serum amyloid A protein, α2-macroglobulin, α1-antitrypsin, α1-antichymotrypsin, and transthyretin. These findings emphasize the significance of inflammatory biomarkers in CSF for the early and accurate diagnosis of meningitis, which is crucial for timely intervention and improved patient outcomes (Belogurov et al. 2016; Hegen and Deisenhammer 2009).

Biosensors, microfluidics, and LAMP are transforming point-of-care testing devices which help in the detection of meningitis-associated biomarkers from non-CSF samples. LAMP assays on nasopharyngeal specimens exemplify non-invasive strategies, bypassing lumbar puncture while maintaining sensitivity. Blood-based biomarkers, such as cortisol and IL-6, are being validated for their diagnostic equivalence to CSF markers, offering safer alternatives in resource-limited settings (Bourke et al. 2010).

In conclusion, emerging technologies like NGS, Multiplex PCR, and biomarker-based detection from alternative sample sources are set to transform the landscape of meningitis diagnosis. Continued research and clinical validation are crucial to ensuring adoption. The implementation of non-invasive molecular diagnostic techniques can significantly reduce diagnostic delays, improve patient outcomes, and minimize the risk associated with traditional invasive procedures. As these technologies continue to evolve, integration with artificial intelligence and machine learning-based predictive models may further improve diagnostic accuracy and efficiency, paving the way for more personalized and timely therapeutic interventions in meningitis management.

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Chapter 8
Trends in Meningitis Prevalence and the Need for Molecular Diagnosis

Mirza Sarwar Baig, Aman Haidar Rizvi, Sanchit Varma, Kaify Ghaus, Alisha Arzoo, Saloni, and Mohammad Anas

Abstract Meningitis is a devastating and potentially life-threatening disease caused by the inflammation of the meninges, which are the membranes that surround the brain and the spinal cord. The inflammation is usually caused by an infection of the fluid surrounding the brain and the spinal cord, known as cerebrospinal fluid (CSF). This inflammation can be caused by infection with viruses, bacteria, fungi, or parasites and sometimes by non-infectious causes such as certain medications or autoimmune diseases. Meningitis is a major contributor to global illness and death, particularly among newborns (neonates) and young children. The disease's impact varies depending on geographic location and the causative organism. Mortality rates range from 10% to 15%, with incidence rates reported between 0.25 and 6.1 per 1000 live births worldwide—exceptionally low at 0.3 per 1000 in developed countries, moderate at 0.48–2.4 per 1000 in Asia, and high at 0.81–6.1 per 1000 in Africa and South Africa. In 2010, an estimated 7.6 million deaths occurred among children under five, with 64.0% (4.879 million) due to infectious causes and 40.3% (3.072 million) occurring in newborns. Of these neonatal deaths, 5.2% (0.393 million) were attributed to sepsis or meningitis.

Keywords Meningitis · Viral meningitis · Pathogenesis · Molecular diagnosis · Therapeutics · PCR · Vaccine

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8.1 Introduction

Meningitis is a devastating and potentially life-threatening disease caused by the inflammation of the meninges, which are the membranes that surround the brain and the spinal cord. The inflammation is usually caused by an infection of the fluid surrounding the brain and the spinal cord, known as cerebrospinal fluid (CSF). This inflammation can be caused by infection with viruses, bacteria, fungi, or parasites and sometimes by non-infectious causes such as certain medications or autoimmune diseases. Meningitis is a major contributor to global illness and death, particularly among newborns (neonates) and young children. The disease's impact varies depending on geographic location and the causative organism. Mortality rates range from 10% to 15%, with incidence rates reported between 0.25 and 6.1 per 1000 live births worldwide—exceptionally low at 0.3 per 1000 in developed countries, moderate at 0.48–2.4 per 1000 in Asia, and high at 0.81–6.1 per 1000 in Africa and South Africa. In 2010, an estimated 7.6 million deaths occurred among children under five, with 64.0% (4.879 million) due to infectious causes and 40.3% (3.072 million) occurring in newborns. Of these neonatal deaths, 5.2% (0.393 million) were attributed to sepsis or meningitis.

Although viruses are the most common causative agents of meningitis, several viruses, including Enteroviruses, Herpesviruses, Influenza viruses, and Arboviruses, can cause viral meningitis, also known as aseptic meningitis. It can affect people of any age and needs emergency medical attention since it might be lethal and lead to serious complications like brain damage, hearing loss, and even death if not treated promptly. This chapter will explore various aspects of meningitis, deep-dwelling into its molecular diagnosis, and address the existing challenges associated with viral meningitis. Our discussion will encompass the latest diagnostic techniques, the complexities of identifying viral pathogens, and the ongoing efforts to overcome hurdles in the field.

8.2 Types of Meningitis

8.2.1 Bacterial Meningitis, aetiology, and symptomology

Bacterial meningitis is undoubtedly one of the most dangerous types of meningitis. Bacteria that lead to meningitis typically make their way into the CNS through the bloodstream after some unfortunate trauma or because of bacterial toxins. It is also quite fascinating that patients with head injuries or those who have cochlear implants (electronic devices they place in the inner ear) are certainly at a higher risk of developing meningitis. Since bacteria can spread from the upper respiratory tract, like the nasopharynx, oropharynx, sinuses, and middle ear, to the CNS through the bloodstream, therefore, bacteria, in their infinite simplicity, can colonize and cross through the mucous membranes of the oropharynx and nasopharynx, effortlessly

entering the bloodstream. Once they find themselves in the blood, these pathogens can spread throughout the body with astonishing ease and can both establish an infection and provoke inflammation in any body site, including the ever-so-sensitive meninges.

Meningitis can be broadly classified into two main types: septic and aseptic meningitis. Septic meningitis, also known as bacterial meningitis (BM), is caused by pus-producing bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* (Tunkel et al. 2004; Young and Thomas 2018). This specific form of meningitis is defined by severe inflammation of the meninges, particularly the arachnoid and pia mater, caused by bacterial invasion and replication in the subarachnoid space (Flexner 1907). The presence of highly active leukocytes in the CSF is a defining feature of bacterial meningitis. The inflammatory process generally spreads beyond the meninges surrounding the brain to impact the brain parenchyma (meningoencephalitis), the ventricles (ventriculitis), and even the spinal cord (Swartz 1984; Kastenbauer et al. 2001). Some investigations have identified neuronal injury, particularly in hippocampal regions, as a possible reason for survivors' chronic cognitive abnormalities (Zysk et al. 1996; Nau et al. 1999). Furthermore, research has shown that bacterial toxins (pneumolysin from pneumococcus, hemolysin, cytolysin) and the cytotoxic products of the host's inflammatory response (H_2O_2, ROS) can cause widespread neuronal loss and synaptic damage, increasing cognitive deficiencies and long-term neurological consequences (Hoffman and Weber 2009; Mook-Kanamori et al. 2011; Grandgirard et al. 2013; Kloek et al. 2020). Human histopathological data indicated that parenchymal damage resulted from elevated intracranial pressure (ICP), cytotoxic and vasogenic oedema, herniation, and local leukocyte infiltration or abscess formation. Additionally, cortical necrosis and hippocampal neuronal loss contributed to the observed damage (Vergouwen et al. 2010). Furthermore, inflammatory indicators, including concentrations of the pro-inflammatory cytokines (IL-1β and TNF-α) in the CSF, are being studied as potential biomarkers for the severity and prognosis of BM (Barichello et al. 2016). Bacterial meningitis requires prompt diagnosis and treatment to avoid life-threatening complications and limit long-term neurological damage.

8.2.2 Meningococcal Meningitis (MM)

Meningococcal meningitis (MM) is a serious infection brought on by the notorious gram-negative coccus *N. meningitidis*. This bacterium has quite a penchant for mucosal membranes in the oropharynx and nasopharynx. Contact with respiratory secretions that contain *N. meningitidis* is an exceptionally effective way to spread this infection. In a shocking twist, death can happen within mere hours after symptoms appear. Nonfatal cases, however, can lead to irreversible nerve damage, which might result in hearing loss and brain damage or even the amputation of limbs due to tissue necrosis. MM constitutes a serious health concern that can impact

individuals irrespective of age, although it is particularly intriguing that its occurrence is most pronounced among infants, adolescents, and young adults (CDC 2024; Pugazenthi et al. 2024).

This MM was historically the predominant etiological agent of meningitis epidemics within human populations. While it continues to wreak havoc in a delightful region of sub-Saharan Africa known as the meningitis belt, it has become quite rare in most other areas, all thanks to those miraculous meningococcal vaccines. In 2010, countries in Africa's meningitis belt began utilizing a new serogroup A meningococcal conjugate vaccine. This program has, quite astonishingly, led to a dramatic reduction in cases of meningococcal meningitis by bestowing both individual and herd immunity.

The pathogenicity of *N. meningitidis* is, of course, enhanced by a plethora of virulence factors that contribute to the rapid progression of this delightful disease. These include lipooligosaccharide (LOS) endotoxin, type IV pili for charmingly attaching to host tissues, and polysaccharide capsules that cleverly aid the cells in evading phagocytosis and complement-mediated killing. Additional virulence factors, such as IgA protease (which dismantles IgA antibodies), the invasion factors Opa, Opc, and porin (which facilitate the transcellular entry through the blood-brain barrier), iron-uptake factors (that efficiently strip heme units from haemoglobin in host cells for their growth), and stress proteins that protect bacteria from those pesky reactive oxygen molecules.

An exceptionally unique hallmark of meningococcal meningitis is the occurrence of a recognizable petechial rash on the skin or mucosal surfaces, characterized by minuscule, red, flat, haemorrhagic lesions. This delightful rash, which appears shortly after disease onset, responds to the LOS endotoxin and adherence virulence factors that disrupt the endothelial cells of capillaries and small veins in the skin. The disruption of blood vessels triggers the formation of tiny blood clots, leading to blood leaking into the surrounding tissue. As the infection progresses, the levels of virulence factors increase, allowing the haemorrhagic lesions to grow larger as blood continues to escape into tissues. Lesions larger than 1.0 cm typically occur in patients developing shock, as those ever-so-helpful virulence factors cause increased haemorrhage and clot formation. Sepsis, resulting from the systemic mayhem caused by meningococcal virulence factors, can lead to rapid multiple-organ failure, shock, disseminated intravascular coagulation, and, of course, death.

Because meningococcal meningitis progresses at a dizzying pace, a greater variety of clinical specimens are required for the timely detection of *N. meningitidis*. Required specimens include blood, CSF, naso- and oropharyngeal swabs, urethral and endocervical swabs, petechial aspirates, and biopsies. MM is diagnosed by directly examining CSF with a Gram stain, which reveals extracellular and intracellular gram-negative diplococci with a unique coffee-bean microscopic appearance consistent with polymorphonuclear neutrophils (PMNs). Latex agglutination and immunochromatographic fast diagnostic techniques specific to *N. meningitidis* can also identify the organism directly in CSF. DNA sequence-based typing approaches for *N. meningitidis* hypervariable outer membrane proteins can also identify species, replacing sero-(sub)-typing.

Meningococcal infections are treatable with antibiotics, with third-generation cephalosporins being the most used. However, preventative immunization is the most effective therapeutic option because outcomes can be unfavourable even with treatment. *N. meningitidis* has 12 recognized capsular serotypes, among which A, B, C, W, X, and Y are the most common globally. The CDC recommends that children aged 11–12 receive a single dose of a quadrivalent vaccination that protects against serotypes A, C, W, and Y, with a booster at age 16 (US-CDC, Recommended Immunization Schedule for Persons Aged 0–18 Years). Individuals in high-risk settings may receive additional booster or serogroup B meningococcal vaccination doses (CDC 2024).

8.2.3 Pneumococcal Meningitis (PM)

The encapsulated gram-positive bacteria *S. pneumoniae* (also known as pneumococcus) causes pneumococcal meningitis (PM). This bacterium is widely found in the pharyngeal microbiota of 30–70% of young children but is present in less than 5% of healthy adults. This bacterium can pass the blood-brain barrier (BBB) in vulnerable individuals, although it is frequently present in non-symptomatic adults. In some situations, it may also cause septicaemia. Since the introduction of the *H. influenzae* serotype b (Hib) vaccination, *S. pneumoniae* has become the primary cause of meningitis in persons aged 2 months to maturity.

The major virulence factors *S. pneumoniae* produces include PI-1 pilin for pneumococcal adherence to host cells and virulence factor B (PavB) for attachment to respiratory tract cells. They also produce choline-binding proteins (cbpA) that bind to epithelial cells and interfere with immune factors immunoglobulin A (IgA) and complement factor 3 (C3)—meanwhile, the cytoplasmic bacterial toxin pneumolysin which instigates an inflammatory response.

S. pneumoniae can be detected in CSF samples utilizing latex agglutination, gram-stained specimens, and an immunochromatographic RDT specific for *S. pneumoniae*. Gram-stained samples show S. pneumoniae as gram-positive, lancet-shaped diplococci. CSF and blood cultures can also be used to identify *S. pneumoniae*, and the quellung reaction to distinctive capsular polysaccharides can reveal about 93 different serotypes. Identification can also be confirmed using PCR and RT-PCR techniques.

To treat pneumococcal meningitis caused by drug-resistant *S. pneumoniae* strains, broad-spectrum medicines such as cefotaxime, levofloxacin, penicillin, or other β-lactam antibiotics are commonly used. Pneumococcal conjugate vaccine (PCV13) and pneumococcal polysaccharide vaccine (PPSV23) are the two pneumococcal vaccines that are currently on the market. These are typically administered to the most vulnerable groups of people: children under the age of 2 and adults over the age of 65.

8.2.4 *Haemophilus Meningitis (HM)*

Meningitis resulting from the encapsulated pleomorphic gram-negative coccobacilli *H. influenzae* serotype b (Hib) has become relatively rare in numerous countries, mainly attributable to the widespread administration of the effective Hib vaccine. In the absence of Hib vaccination, *H. influenzae* can serve as the principal etiological agent for meningitis among children aged 2 months to 5 years. This pathogen is commonly present in the throats of otherwise healthy individuals, including infants and young children. By age five, most children have developed immunity to this organism. However, infants older than 2 months lack a sufficiently robust protective antibody response, rendering them vulnerable to severe disease. The elevated intracranial pressure associated with this infection results in a mortality rate of 5% and a 20% incidence of deafness or neurological impairment among survivors (US-DHHS 2019).

H. influenzae is known to produce a minimum of 16 distinct virulence factors. For instance, the Haemophilus adhesion and penetration factor (Hap) facilitates attachment and invasion of respiratory epithelial cells, and the lipooligosaccharide (LOS) instigates inflammation. This bacterium is also equipped with a polysaccharide capsule that aids in evading phagocytosis, alongside factors such as P2 protein and IgA1 protease that enable it to circumvent antibodies produced by mucous membranes. Furthermore, factors such as transferrin-binding protein (Tbp) and haemoglobin-binding protein (Hgp) are utilized to extract iron from transferrin and haemoglobin, respectively, thereby supporting bacterial proliferation.

The preliminary diagnosis of *H. influenzae* infections can be conducted through direct smear analysis of cerebrospinal fluid (CSF) testing. Stained smears typically reveal intracellular and extracellular polymorphonuclear leukocytes (PMNs) alongside small, pleomorphic, gram-negative coccobacilli or filamentous forms characteristic of *H. influenzae*. Morphological confirmation of this bacteria can be established based on its fastidious growth on chocolate agar. Definitive identification is corroborated by the necessity for exogenous biochemical growth cofactors NAD and heme (via MALDI-TOF), latex agglutination, PCR and reverse transcription PCR (RT-PCR).

Meningitis induced by *H. influenzae* is generally treated with a regimen that includes doxycycline, fluoroquinolones, 2nd and 3rd generation cephalosporins, and carbapenems. The most effective strategy for preventing *H. influenzae* infection involves the administration of the Hib polysaccharide conjugate vaccine. All children are advised to receive this vaccine at 2, 4, and 6 months of age, followed by a final booster dose administered between 12 and 15 months of age (CDC 2024).

8.2.5 *Neonatal Meningitis (NM)*

Newborns are particularly susceptible to infection due to their immature cellular and humoral immune systems, including underdeveloped phagocytic function (Pong and Bradley 1999; Filias et al. 2011). A full-term newborn's innate immune system is distinct, leaning towards T-helper type 2/T-helper type 17 polarization and the production of anti-inflammatory cytokines and, in contrast, showing impaired T-helper type 1 polarization and cytokine production. The limited expression of complement, along with antimicrobial proteins and peptides, contributes to a newborn's vulnerability to pyogenic bacteria (Cuenca et al. 2013). The blood-brain-barrier (BBB) development begins in late gestation and persists into the postnatal period, during which increased permeability may make the developing brain more vulnerable (Daneman et al. 2010; Coisne and Engelhardt 2011; Mook-Kanamori et al. 2011). Research has also shown that tight junctions are mechanistically and functionally effective in the embryonic brain. Some transporters are more active during development than in the adult brain (Daneman et al. 2010; Mook-Kanamori et al. 2011; Gordon et al. 2017; Bundy et al. 2024). Long-term neurological complications, such as deafness, blindness, cerebral palsy, seizures, hydrocephalus, or cognitive impairment, occur in 25–50% of survivors (de Louvois et al. 2005).

Streptococcus agalactiae, classified as a Group B Streptococcus (GBS), is an encapsulated Gram-positive bacterium recognized as the predominant etiological agent of neonatal meningitis in infants aged up to 3 months (Thigpen et al. 2011). *S. agalactiae* is also capable of causing meningitis across all age demographics and is present in the urogenital and gastrointestinal microbiota of approx. 10–30% of the human population.

Neonatal *S. agalactiae* infection can manifest as either early-onset or late-onset meningitis. Early-onset meningitis is characterized by its occurrence in infants up to 7 days old. The initial infection by *S. agalactiae* typically transpires during the parturition process when the bacteria may be transmitted from the mother's vaginal flora. The incidence of early-onset neonatal meningitis can be significantly diminished through the administration of intravenous antibiotics to the mother during labour. Late-onset neonatal meningitis is identified in infants aged between 1 week and 3 months. Infants born to mothers harbouring *S. agalactiae* in the urogenital tract exhibit an increased susceptibility to late-onset meningitis. However, late-onset infections may arise from sources other than the mother, with the specific source of infection frequently remaining unidentified. Additionally, infants born prematurely (before 37 weeks of gestation) or to mothers who experience febrile episodes are at an elevated risk of developing late-onset neonatal meningitis.

Clinical manifestations of early-onset disease encompass temperature instability, apnea (temporary cessation of breathing), bradycardia (decreased heart rate), hypotension, feeding difficulties, irritability, and decreased muscle tone. During sleep, the infant may exhibit difficulty in arousal. Symptoms associated with late-onset disease are more likely to present as seizures, bulging fontanelle (soft spot), nuchal rigidity (stiff neck), hemiparesis (weakness on one side of the body), and

opisthotonos (a condition characterized by a rigid body with an arched back and the head thrown backwards).

S. agalactiae produces at least 12 virulence factors, including FbsA, which facilitates adhesion to host cell surface proteins. These PI-1 pili enhance the invasion of human endothelial cells, a polysaccharide capsule that obstructs the activation of the alternative complement pathway and impedes phagocytosis, and the CAMP factor toxin, which creates pores in host cell membranes while binding to IgG and IgM antibodies.

The diagnosis of neonatal meningitis is frequently, though not consistently, affirmed through positive cultures from cerebrospinal fluid (CSF) or blood. Diagnostic tests encompass routine cultures, antigen detection via enzyme immunoassay, serotyping of various capsule types, polymerase chain reaction (PCR), and reverse transcription PCR (RT-PCR). Neonatal meningitis is treated with β-lactam antibiotics, such as intravenous penicillin or ampicillin, in conjunction with gentamicin. Despite therapeutic interventions, an approximate mortality rate of 10% is observed among infected neonates (Heath et al. 2004).

8.3 Viral Meningitis, aetiology and symptomology

Although viral meningitis or aseptic meningitis is far more prevalent than its bacterial counterpart, it manifests with a milder intensity. Different viruses can cause meningitis, including arboviruses, enteroviruses, adenovirus, lymphocytic choriomeningitis virus (LCMV), influenza, parainfluenza, and many more. A myriad of viruses can trigger meningitis because of the initial infection, encompassing those responsible for herpes, measles, and mumps. Meningitis or encephalitis may emerge as secondary sequelae associated with a multitude of viral agents or may be instigated by arboviruses (arthropod-borne). The resultant diseases are categorized under arboviral encephalitis (Table 8.1). Most arboviruses exhibit endemism within geographical boundaries. Enteroviruses are also the leading cause of aseptic meningitis, but other viruses such as herpes simplex virus (HSV), mumps virus, and West Nile virus (WNV) can also be responsible (Rotbart and Webster 2001; Logan and MacMahon 2008; Noska et al. 2015). Viral meningitis occurs throughout the year but is most seen in summer and fall (Table 8.1).

HIV is an important yet overlooked cause of viral meningitis, diagnosed in stored CSF samples from 57 patients from North Carolina who had presented with non-bacterial meningitis were retrospectively tested positive for HIV RNA (Hanson et al. 2008; Vigil et al. 2018). Not all patients with aseptic meningitis have viral meningitis; therefore, it is crucial to consider other causes if molecular assays do not confirm viral aetiology. Unlike septic meningitis, aseptic meningitis generally has a more benign course, with patients often presenting with similar symptoms of fever, headache, and neck stiffness but with a tendency towards a milder illness and lower risk of severe complications (Kupila et al. 2006). Despite the less severe

Table 8.1 List of viral infections causing meningitis or encephalitis

Viruses	Season of occurrence	Diagnosis	Therapeutics
Arboviruses	Summer and Fall	CSF and serum for antibody detection	The vaccine is available only for JEV, and there are no vaccines or antiviral drugs for other arboviruses
Enteroviruses	Summer and Fall	Testing of oral, faecal, throat/ nasopharynx, CSF, and urine samples	Antiviral drugs and vaccines are available for some enteroviruses
Herpes simplex virus (HSV)	Year-around	Brain biopsy, blood serum, or CSF samples and PCR detection	Nucleoside analogue drugs are specific to herpes infection. No vaccine is available
Lymphocytic choriomeningitis virus (LCMV)	Year-around	Serum for antibody detection	No antiviral agents have undergone clinical trials for the treatment of LCMV infection. Vaccination with a recombinant LCMV-based vector is available
Mumps virus (MuV)	Year-around	Serum for antibody detection	MMR vaccine is available as part of the routine immunization schedule
HIV	Year-around	Brain biopsy and detected by culture/ PCR	More than 20 antiviral drugs are available for treatment of HIV infections
JC Polyomavirus (JCPyV)	Year-around	Brain biopsy and detected by EM/PCR	There is no specific antiviral drug against JCPyV, but antiretroviral drugs are used for HIV patients and acute infection
Rabies virus (RABV)	Year-around	Corneal cells, brain	Antiviral drugs and vaccines are available for RABV

presentation, accurate diagnosis and differentiation from bacterial meningitis are crucial for appropriate management and treatment (Table 8.1).

8.4 Epidemiology of Viral Meningitis

8.4.1 Epidemiology of Viral Meningitis in Children

The epidemiology of viral meningitis in children varies worldwide, with notable differences in incidence rates, common causative viruses, and clinical manifestations. In Denmark, between 1977 and 2001, there were 1642 reported cases, with an incidence of 38.7 per 100,000 among 6-month-old infants. The primary causative viruses were mumps and non-polio enteroviruses. In Fiji, from 2004 to 2007, 12 cases were identified, accounting for 17.1% of the total cases, with enteroviruses as

the common cause and fever being the predominant clinical symptom. In the USA, from 2005 to 2011, there were 7618 cases of viral meningitis, primarily caused by enteroviruses, with common symptoms including fever and headache. In China, in 2013, 74 cases were recorded, with enterovirus 71 (EV-71) being the primary causative agent, and clinical presentations often included fever and myoclonic seizures. In England, between 2014 and 2015, 703 cases were documented, with enteroviruses and human parechoviruses identified as the common pathogens. The incidence rates were 0.79 per 1000 for enteroviruses and 0.04 per 1000 for human parechoviruses, with fever and irritability being the most frequent symptoms (Fig. 8.1a and Table 8.2). Denmark reported the highest incidence rate at 38.7 per 100,000 in 6-month-old infants, highlighting a significant burden among young children. Non-polio enteroviruses and mumps were prevalent in Denmark, while

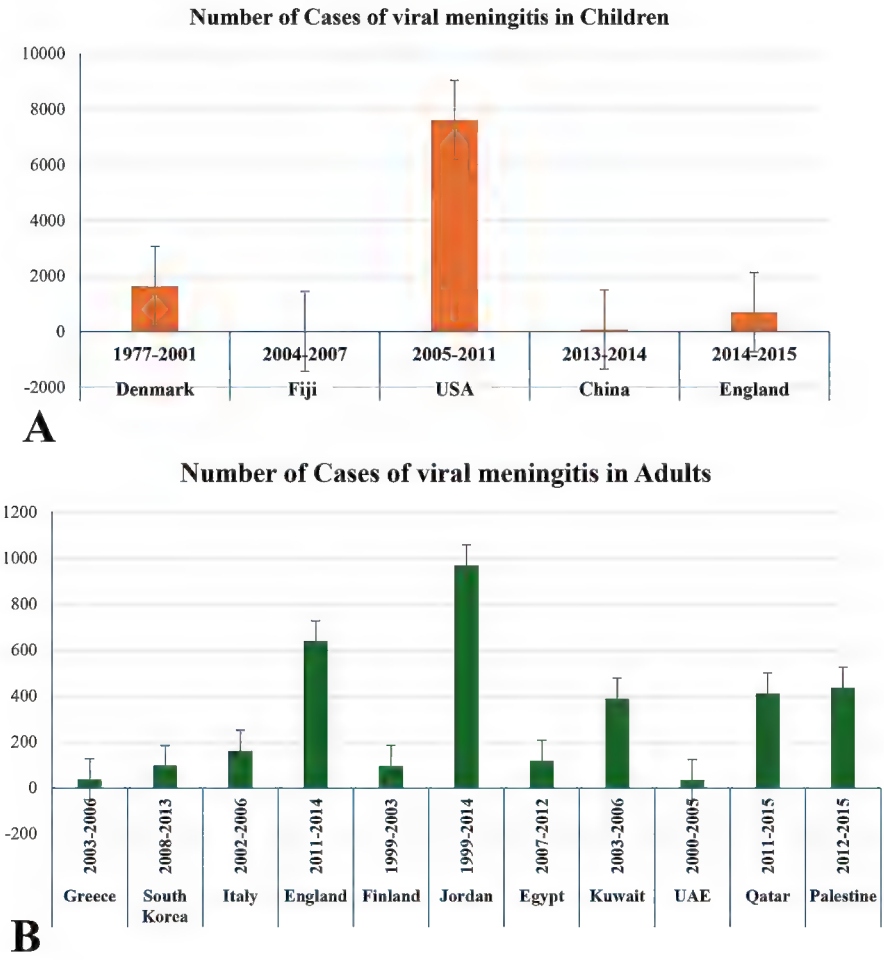

Fig. 8.1 Worldwide epidemiological distribution of viral meningitis in (**a**) children and (**b**) adults

Table 8.2 Epidemiological data of viral meningitis worldwide

Country	Years	Number of cases	Common causative virus	Clinical manifestation	References
Viral meningitis in children					
Denmark	1977–2001	1642	Mumps and non-polio enteroviruses (EV)	–	Hviid and Melbye (2007)
Fiji	2004–2007	12	Enterovirus (EV)	Fever	Biaukula et al. (2012)
USA	2005–2011	7618	Enterovirus (EV)	Fever and headache	Nigrovic et al. (2013)
China	2013–2014	74	Enterovirus 71 (EV-71)	Fever and myoclonic seizures	Hu et al. (2015)
England	2014–2015	703	Enterovirus (EV) and Human Parechovirus (HPeV)	Fever and irritability	Kadambari et al. (2020)
Viral meningitis in adults					
Greece	2003–2006	36	EV, VZV, and HSV-2	–	Frantzidou et al. (2008)
South Korea	2008–2013	96	EV and VZV	Headache and fever	Han et al. (2016)
Italy	2002–2006	162	–	Fever and headache	Magazzini et al. (2012)
England	2011–2014	638	Non-polio enteroviruses	Headache and photophobia	McGill et al. (2018)
Finland	1999–2003	95	EV	Headache and photophobia	Kupila et al. (2006)
Jordan	1999–2014	969	E-9 and Coxsackievirus B4	Fever and vomiting	Meqdam et al. (2002), Alzein 2016)
Egypt	2007–2012	117	EV and HSV	Fever and signs of meningeal irritations	Selim et al. (2007), Shaker and Abdelhamid (2015)
Kuwait	2003–2006	387	E-9, E-11, and E-30	Fever	Dalwai et al. (2010)
UAE	2000–2005	34	–	Fever and neck stiffness	Dash et al. (2007)
Qatar	2011–2015	411	EV	Fever and headache	Ben Abid et al. (2018)
Palestine	2012–2015	436	EV	Fever and vomiting	Ghuneim (2016), Dumaidi and Al-Jawabreh (2017)

enteroviruses were the predominant cause across Fiji, the USA, and China. In England, both enteroviruses and human parechoviruses were common causative agents. Fever was a consistent symptom across all regions. Additional symptoms included headache in the USA, myoclonic seizures in China, and irritability in England. The data demonstrate geographical variations in the incidence, causative viruses, and clinical presentations of viral meningitis among children. This underscores the importance of region-specific public health strategies for prevention and management.

8.5 Epidemiology of Viral Meningitis in Adults

The epidemiology of viral meningitis in adults shows significant variations globally, with differences in the number of cases, causative agents, and clinical manifestations across various countries. In Greece, from 2003 to 2006, there were 36 reported cases, with enteroviruses (EV), varicella-zoster virus (VZV), and herpes simplex virus type 2 (HSV-2) being the primary causative agents. In South Korea, between 2008 and 2013, 96 cases were reported, with 54.2% caused by enteroviruses and VZV; common symptoms included headache and fever. From 2002 to 2006, there were 162 cases in Italy, with 80% presenting with fever and headache, although specific causative agents were not detailed. In England, from 2011 to 2014, 638 cases were reported, with non-polio enteroviruses being responsible for 57% of the cases, and common clinical manifestations included headache and photophobia. In Finland, between 1999 and 2003, there were 95 cases, with 66% caused by enteroviruses, and patients commonly experienced headaches and photophobia (Fig. 8.1b and Table 8.2).

The epidemiology of viral meningitis in the Middle East and North Africa (MENA) region demonstrates notable variability in the number of cases, causative agents, and clinical manifestations across different countries. Several outbreaks have been reported in Jordan, with 32 cases in 1999 primarily caused by Echovirus 9 (E-9) and Coxsackievirus B4, presenting with fever and vomiting. In subsequent years, Jordan reported 474 cases in 2001 and 463 cases in 2014, with fever being the most common clinical manifestation, although specific causative agents were not identified. In Egypt, there were 17 cases (6.8%) in 2007 associated with enteroviruses (EV) and herpes simplex virus (HSV), while from 2010 to 2012, 100 cases were caused by EV, presenting with fever and signs of meningeal irritation (Fig. 8.1b and Table 8.2). Kuwait recorded 387 cases of viral meningitis between 2003 and 2006, primarily due to E-9, E-11, and E-30, with fever being the most frequently reported symptom. In the United Arab Emirates (UAE), 34 cases (37%) were reported from 2000 to 2005, with fever and neck stiffness being common clinical manifestations, although the causative agents were not specified. Qatar observed 411 cases (74.7%) between 2011 and 2015 caused by enteroviruses, with patients commonly experiencing fever and headache. In Palestine, 80 cases (62%) were reported from 2013 to 2014, mainly due to enteroviruses, with fever and vomiting

as common symptoms. From 2012 to 2015, Palestine reported 356 cases associated with Enterovirus B (EV-B), where fever and headache were frequently observed clinical manifestations (Fig. 8.1b and Table 8.2).

Overall, enteroviruses are the predominant causative agents of viral meningitis in these regions, with fever, headache, vomiting, and signs of meningeal irritation being the most reported clinical symptoms. This comparative data highlights the prevalence of enteroviruses as a common causative agent of viral meningitis in adults worldwide, with headache and fever being the most frequently observed clinical symptoms. These data highlight significant regional variations in the incidence, causative viruses, and clinical manifestations of viral meningitis among children, emphasizing the need for targeted public health interventions based on regional epidemiological patterns.

8.6 Clinical Manifestations

Early symptoms of bacterial meningitis include fever, fatigue, and headaches. As the condition advances, meningeal irritation symptoms emerge, including neck stiffness (meningismus), photophobia, phonophobia, and vomiting (van de Beek et al. 2004). Headache and meningismus are symptoms of inflammatory activation of trigeminal sensory nerve fibres in the meninges. Less than half of individuals with diagnosed bacterial meningitis experience the usual triad of fever, neck stiffness, and altered mental state. Approximately one-third of patients suffer focal neurological symptoms, such as epileptic seizures or limb paresis, and up to 69% experience decreased awareness or coma in 14% of instances (van de Beek et al. 2004).

Viral meningitis can be misdiagnosed because its signs and symptoms are frequently confused with those of other illnesses, such as encephalitis or brain abscesses. Fever, chills, nausea, headaches, and stomach discomfort are common symptoms in patients with viral meningitis (Kumar 2005). Additional typical symptoms include sensitivity to bright light, neck pain and stiffness, loss of appetite, and fast breathing (Parikh et al. 2012). Patients may also have double vision and have trouble concentrating or focusing; these symptoms may not go away even after they recover (Irani 2008).

Clinical features or symptoms alone are insufficient in situations of suspected meningitis to differentiate between other diagnoses, such as bacterial and non-bacterial etiologies. Cerebrospinal fluid (CSF) analysis is so crucial. Examining the CSF's appearance and opening pressure (cm H_2O), CSF leukocyte counts (cells/μL), CSF protein levels (g/L), predominant cell type in CSF, and CSF-to-blood glucose ratio are all common components of CSF analysis. In healthy adults, CSF typically has an opening pressure of $\leq$20 cm H_2O and leukocyte counts ranging from 0 to 5 cells/μL. The primary cell type in normal CSF is lymphocytes, with protein levels ranging from 0.15 to 0.45 g/L. Furthermore, the CSF-to-blood glucose ratio remains around 0.6, showing that glucose levels in the CSF are normal

when compared to blood. These parameters offer crucial data for precise diagnosis and suitable treatment.

In contrast, bacterial meningitis causes significantly aberrant CSF outcomes. Patients with bacterial meningitis frequently have an increased opening pressure of more than 20 cm H_2O. The CSF leukocyte count increases dramatically, often ≥1000 cells/μL, with neutrophils being the predominant cell type. This represents the immediate inflammatory response to bacterial infection. Protein levels in CSF reach ≥1.0 g/L due to increased blood-brain barrier (BBB) permeability because of inflammation (Tunkel et al. 2015). Furthermore, the CSF-to-blood glucose ratio decreases to ≤0.5, indicating a decreased glucose concentration in the CSF due to bacterial consumption and disruption of glucose transport during inflammation.

Viral meningitis, on the other hand, produces CSF results that are distinct from normal and bacterial meningitis. The opening pressure in viral meningitis is often ≤20 cm H_2O, similar to healthy persons. However, the CSF leukocyte count is fewer than 1000 cells/μL, with lymphocytes being the primary cell type after the initial 48 h, where neutrophils may dominate. This pattern represents the body's immune response to viral infections involving lymphocytes. The CSF-to-blood glucose ratio is generally around 0.6, comparable to normal levels because viral infections do not utilize glucose in the same manner as bacterial infections do. The elevation in protein concentration is generally more pronounced in bacterial meningitis (≥1.0 g/L) compared to viral meningitis (<1.0 g/L). However, in meningitis caused by herpes simplex virus (HSV) or varicella-zoster virus (VZV), CSF protein levels may also be ≥1 g/L (Kaewpoowat et al. 2016).

8.7 Pathogenesis of Bacterial Meningitis

Meningitis pathogen-associated molecular patterns (PAMPs) such as lipoprotein (LP), lipoteichoic acid (LTA), peptidoglycan (PG), and lipopolysaccharide (LPS) are recognized by host immune pattern recognition molecules like CD14 and LBP, which function as sensors (Beutler 2003; Ivey et al. 2005; Hoffmann et al. 2007; Hoffman and Weber 2009). Due to high-grade bacteremia, bacteria can invade the central nervous system (CNS) either from the bloodstream or through direct access routes such as dural defects or local infections, leading to meningitis. Meningitis is characterized by a rapid inflammatory response, which is caused by many inflammatory signalling cascades, including NFkB and MAP kinases, which are triggered by TLR signals and conveyed via the intracellular adaptor protein MyD88 (Lehnardt et al. 2006).

According to experimental data, bacterial invasion of the choroid plexus may have occurred. In humans, the Toll-like receptor (TLR) family consists of 11 members, which can be divided into two main groups: one group is found on the cell surface and detects extracellular ligands, while the other is present in the endosomes and recognizes pathogen nucleic acids (Hanke and Kielian 2011). Microglia cells express TLRs 1–9, while astrocytes express TLRs 2, 3, and 9. Neurons express TLRs 3, 7, 8, and 9, while oligodendrocytes express TLRs 2 and 3 (Table 8.3).

Table 8.3 Interaction of different categories of bacterial PAMPs (as TLR Agonist) with human Toll-like receptors (TLRs) essential for their pathogenesis, promoting bacterial survival and virulence

Bacteria	TLR agonist	TLR agonist activates immune cells of the human central nervous system (CNS) by different Toll-like receptors
Streptococcus pneumoniae	Peptidoglycan (PG)	recognized by TLR-2 in combination with peptidoglycan recognition protein (PGRP) and CD14
	Lipoteichoic acid (LTA)	recognized by TLR-2 in combination with Lipopolysaccharide-binding Protein (LBP), and CD14
	Pneumolysin (bacterial toxins)	recognized by TLR-4
	Bacterial CpG motifs	recognized by TLR-9
	Triacylated lipoprotein (TLP)	recognized by TLR-2 in conjunction with TLR-1
	Diacylated lipoprotein (DLP)	recognized by TLR-2 in conjunction with TLR-6
Neisseria meningitidis	lipooligosaccharide (LOS) a modified Lipopolysaccharide (LPS)	recognized by TLR-4 in combination with coreceptors MD-2 and CD14
	Outer membrane vesicles (OMVs)	strongly recognized by TLR-4 and other TLRs may also be involved
	Outer membrane proteins trimeric-Porins (PorB)	binds to TLR-2
Haemophilus influenzae type b (Hib)	Lipopolysaccharide (LPS)	interacts mainly through the TLR-4 pathway
	Outer membrane vesicles (OMVs)	interacts with human TLR-2
	Triacylated lipoprotein (TLP)	activates the TLR-2/TLR-1 pathway
	Diacylated lipoprotein (DLP)	activates TLR6-TLR2 heterodimers
Streptococcus agalactiae	Lipoteichoic acid (LTA)	Primarily recognized by TLR-2 and CD14.
	Microbial RNA (Particularly single stranded)	interacting with TLR signalling complexes, including TLR-8 and TLR-13
	Triacylated lipoprotein (TLP)	interacts with TLR-2 and TLR-1
	Diacylated lipoprotein (DLP)	interacts with TLR-2 and TLR-6 heterodimers
Mycobacterium tuberculosis (MTB)	Lipoarabinomannan (LAM)	particularly interact with TLR-2
	Bacterial CpG motifs	particularly TLR-9, but TLR2 and TLR9 can cooperate to enhance host resistance
	Microbial RNA	recognized by intracellular TLRs, such as TLR-3, TLR-7, and TLR-8, which are located in endosomes and phagolysosomes

(continued)

Table 8.3 (continued)

Bacteria	TLR agonist	TLR agonist activates immune cells of the human central nervous system (CNS) by different Toll-like receptors
Listeria monocytogenes	Lipoteichoic acid (LTA)	binds to TLR-2 receptor and triggers a signalling cascade that activates immune cells, especially macrophages and dendritic cells
	Flagellin	interacts with TLR-5
	Triacylated lipoprotein (TLP)	interacts with TLR1-TLR2 heterodimers
	Diacylated lipoprotein (DLP)	interacts with TLR6-TLR2 heterodimers
Escherichia coli	Lipopolysaccharide (LPS)	TLR-4 is the primary receptor for LPS, while host receptors like TLR-2 and CD14 also play a role in LPS recognition and signalling
	Triacylated lipoprotein (TLP)	TLR-1 and TLR-2 heterodimer interact with TLP
	Diacylated lipoprotein (DLP)	TLR-2 and potentially TLR-6 interact with DLP

Meningococci have been detected in the meninges and choroid plexus, but meningitis caused by pneumococci infiltrates the leptomeningeal blood vessels (Zwijnenburg et al. 2001). Several studies showed that streptococcal proteins, like CbpA, interact with glycoconjugate receptors of phosphorylcholine with platelet-activating factor (PAF) on eukaryotic cells to promote endocytosis and facilitate the crossing of the blood-brain barrier (Orihuela et al. 2004; Radin et al. 2005). Meningococci's outer membrane protein binds to vitronectin and integrins, and its PilC1 adhesin interacts with CD46 (Unkmeir et al. 2002). Adhesive proteins are present in bacteria that cause infant meningitis, including E. coli and group B streptococcal (GBS), which aid in CNS invasion (Maisey et al. 2007).

TLR2 recognizes pneumococcal PG and LP (Aliprantis et al. 1999; Weber et al. 2003), whereas TLR4 recognizes LPS and pneumococcal toxin pneumolysin signal (Malley et al. 2003). Antibiotic therapy increases the number of bacterial debris, including bacterial DNA, and because of the concentrations of LP, PG, LTA, and LPS in the CSF, it sets off strong inflammatory responses associated with meningitis. By stimulating microglial cells' TLR4 and TLR2 pattern recognition receptors (PRRs), these PAMPs cause neurotoxicity. In the presence of TLR4 and TLR2 receptors on microglia, neuronal death is effectively induced by suitable TLR ligands, reliant on an intact MyD88 pathway (Lehnardt et al. 2003, 2006).

Neuronal damage due to meningitis is multifactorial, including cytotoxic products from immune cells, such as hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), and reactive nitrogen species (RNS), and bacterial toxins, such as pneumolysin, hemolysin, and cytolysin. These factors can result in intracranial

complications (Fig. 8.1). These toxins and cytotoxins cause programmed death of neurons and microglia by inducing rapid mitochondrial damage. Notably, pneumolysin has been shown to translocate to mitochondria and form pores in mitochondrial membranes (Braun et al. 2001, 2007; Bermpohl et al. 2005). The apoptosis-inducing factor (AIF) released from damaged mitochondria leads to large-scale DNA fragmentation and apoptosis-like cell death. This type of cell death is executed in a caspase-independent manner. In a TLR-dependent way, bacterial components activate microglia, triggering the release of cell death signals, such as nitric oxide (NO), to nearby neurons. Highly reactive, tissue-damaging intermediates are created when reactive oxygen and nitrogen species generated from bacteria and hosts mix (Hoffmann et al. 2007). Furthermore, as endogenous danger signals, dying parenchymal cells produce TLR ligands, which initiate an inflammatory tissue damage cycle (Lehnardt et al. 2008). These pathways clarify how neuronal injury is facilitated by active microglia.

Leukocyte influx, characteristic of acute meningitis, can cause neuronal damage and may be more damaging than beneficial to the host. The early inflammatory response and bacterial invasion occur concurrently, with activated leukocyte products such as matrix metalloproteinases (MMPs) and nitric oxide (NO) causing early damage to the blood-brain and blood-CSF barriers (Koedel et al. 1995; Kieseier et al. 1999). Once bacteria penetrate the subarachnoid space, they proliferate, undergo autolysis, and induce further inflammation, resulting in intracranial complications such as brain oedema, vascular changes, hydrocephalus (impairs consciousness), and infarctions. Such factors raise intracranial pressure (ICP) and cause parenchymal damage (Fig. 8.2). Up to 50% of bacterial meningitis survivors experience debilitating cognitive abnormalities because the hippocampus is the most sensitive brain region. Neuronal death frequently causes hippocampal atrophy, as seen in MRI scans of bacterial meningitis survivors.

8.8 Pathogenesis of Viral Meningitis

When the virus enters the host through the fecal-oral pathway or respiratory secretions, it causes an early infection in the gastrointestinal or respiratory tract, which starts the pathogenesis of viral meningitis. Subsequently, viremia causes infection of the central nervous system (CNS), potentially resulting in meningitis or other neurological complications. Multiple processes, such as infection of the lymphoid tissue, infection of the choroid plexus epithelium, induction of inflammation and subsequent breakdown of the blood-brain barrier (BBB), and infection of peripheral sensory neural pathways, can result in viral infection of the CNS (Wong et al. 2008; Kim et al. 2009; Munster et al. 2012). Increased numbers of neutrophils, CD8 T cells, monocytes, and chemo-attractants are seen upon CNS entrance, suggesting the start of an immunological response (Kim et al. 2009). In one study, lymphocytic choriomeningitis virus (LCMV) infection was used as a model for viral meningitis in mice, and raised levels of IL-6 and INF-γ were identified in the CSF. In a different

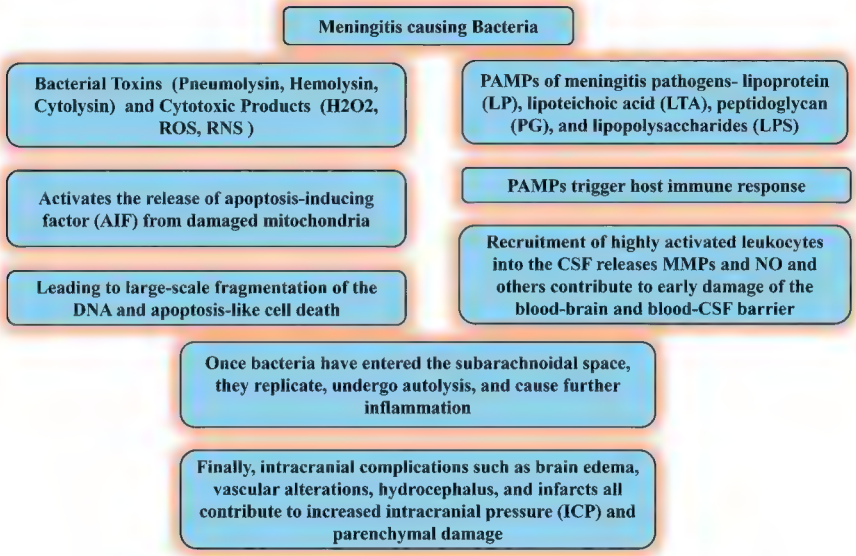
Meningitis causing Bacteria
Bacterial Toxins (Pneumolysin, Hemolysin, Cytolysin) and Cytotoxic Products (H2O2, ROS, RNS)
PAMPs of meningitis pathogens- lipoprotein (LP), lipoteichoic acid (LTA), peptidoglycan (PG), and lipopolysaccharides (LPS)
Activates the release of apoptosis-inducing factor (AIF) from damaged mitochondria
PAMPs trigger host immune response
Recruitment of highly activated leukocytes into the CSF releases MMPs and NO and others contribute to early damage of the blood-brain and blood-CSF barrier
Leading to large-scale fragmentation of the DNA and apoptosis-like cell death
Once bacteria have entered the subarachnoidal space, they replicate, undergo autolysis, and cause further inflammation
Finally, intracranial complications such as brain edema, vascular alterations, hydrocephalus, and infarcts all contribute to increased intracranial pressure (ICP) and parenchymal damage

Fig. 8.2 The flow diagram illustrates the pathogenesis of bacterial meningitis, highlighting two major pathways. The first involves bacterial toxins (pneumolysin, hemolysin, cytolysin) and cytotoxic products such as hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), and reactive nitrogen species (RNS). These factors activate the release of apoptosis-inducing factor (AIF) from damaged mitochondria, leading to large-scale DNA fragmentation and apoptosis-like cell death, which occurs in a caspase-independent manner. The second pathway involves pathogen-associated molecular patterns (PAMPs) of meningitis pathogens, including lipoprotein (LP), lipoteichoic acid (LTA), peptidoglycan (PG), and lipopolysaccharides (LPS). These PAMPs trigger the host immune response, recruiting highly activated leukocytes into the cerebrospinal fluid (CSF). The early inflammatory response and bacterial invasion progress in parallel. Products of activated leukocytes, such as matrix metalloproteinases (MMPs) and nitric oxide (NO), contribute to the early damage of the blood-brain and blood-CSF barriers. Once bacteria enter the subarachnoidal space, they replicate, undergo autolysis, and cause further inflammation. This process leads to intracranial complications, including brain oedema, vascular alterations, hydrocephalus (resulting in impaired consciousness), and infarcts. All these factors contribute to increased intracranial pressure (ICP) and parenchymal damage

study, patients with aseptic meningitis had higher levels of IL-1β, a pro-inflammatory cytokine (Ramilo et al. 1990). Once the virus infiltrates the CNS, an immune response is triggered, characterized by increased levels of chemo-attractants, neutrophils, CD8 T cells, and monocytes. The lymphocytes are the major cell type after the initial 48 h, where neutrophils may dominate (Fig. 8.3).

Additionally, elevated levels of pro-inflammatory cytokines such as IL-6, INF-γ, and IL-1β indicate the intense inflammatory response associated with viral meningitis. The aetiology of aseptic meningitis depends heavily on this strong inflammatory immune response. Due to a lack of data, it is still challenging to understand the pathophysiology of aseptic meningitis since many instances of viral meningitis go undiagnosed and because fatal cases sometimes appear as severe encephalitic

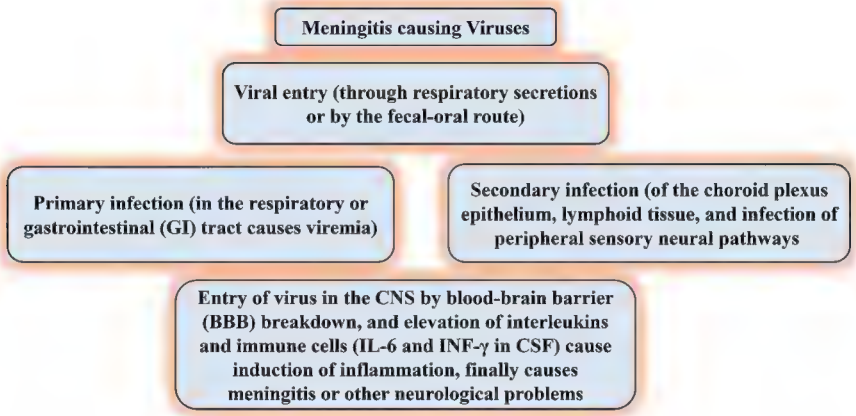
Meningitis causing Viruses
Viral entry (through respiratory secretions or by the fecal-oral route)
Primary infection (in the respiratory or gastrointestinal (GI) tract causes viremia)
Secondary infection (of the choroid plexus epithelium, lymphoid tissue, and infection of peripheral sensory neural pathways
Entry of virus in the CNS by blood-brain barrier (BBB) breakdown, and elevation of interleukins and immune cells (IL-6 and INF-γ in CSF) cause induction of inflammation, finally causes meningitis or other neurological problems

Fig. 8.3 Schematic presentation of the pathogenesis of viral meningitis. This flow diagram illustrates the process by which viruses cause meningitis. The initial stage involves the virus entering the host through the fecal-oral route or respiratory secretions, leading to primary infection in the gastrointestinal or respiratory tract. The virus subsequently invades the central nervous system (CNS), causing secondary infection. Various mechanisms are depicted, including the choroid plexus epithelium infection, lymphoid tissue invasion, inflammation induction resulting in blood-brain barrier (BBB) breakdown, and infection of peripheral sensory neural pathways. Once the virus infiltrates the CNS, an immune response is triggered, characterized by increased levels of chemo-attractants, neutrophils, CD8 T cells, and monocytes. The lymphocytes are the major cell type after the initial 48 h, where neutrophils may dominate. Additionally, elevated levels of pro-inflammatory cytokines such as IL-6, INF-γ, and IL-1β indicate the intense inflammatory response associated with viral meningitis

syndromes rather than meningitis (Irani 2008; Kohil et al. 2021). Further research is needed to develop and improve therapeutic interventions against this disease.

8.9 Different groups of Viruses as an Etiological Agent for Meningitis

Meningitis is the inflammation of the meninges, which means inflammation of the brain and spinal cord membranes, resulting in abnormal cell count in the cerebrospinal fluid (CSF). In viral meningitis, it is the viral infiltration of CNS that elevated levels of pro-inflammatory cytokines such as IL-6, INF-γ, and IL-1β, causing a strong inflammatory response resulting in the inflammation of the meninges. Viral meningitis is most common in young children but is seen across all age groups. Viruses can reach the meninges (CNS) in several ways, including bloodstream viremia from an infection in another part of the body, e.g. throat or lungs, and spreads to the brain or infecting immune cells that carry the virus to the nervous system. Viruses can spread retrogradely from entry at the nerve terminal, and viral particles are transported in axons toward the neuronal cell body from nerve endings and

ultimately to the brain. Viruses can reactivate from a dormant state within the nervous system. As a virus reaches the central nervous system (CNS) and spreads through the subarachnoid space, it causes an inflammatory response, resulting in meningitis. In extreme situations when inflammation occurs in the brain, parenchyma encephalitis is associated with a worse prognosis. Many viruses cause meningitis, and the most common among them are those of the family Picornaviridae.

8.9.1 Arboviruses

Arboviruses (arthropod-borne) of different families have been associated with CNS infection and the development of viral meningitis. These include members of the families *Flaviviridae* (West Nile virus, Zika virus, dengue virus, Japanese encephalitis virus, Saint Louise encephalitis, Powassan), *Peribunyaviridae* (La Crosse virus), and *Togaviridae* (Eastern equine encephalitis virus, Western equine encephalitis virus, chikungunya). Arboviral encephalitis conditions prevalent in the USA are caused by St. Louis encephalitis virus (SLEV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), and West Nile virus (WNV). The geographical dissemination of arboviruses beyond their endemic territories occasionally transpires, typically because of environmental alterations that favour the virus's survival and proliferation or its vector. The augmented mobility of infected humans, animals, or vectors has further facilitated the incursion of arboviruses into new regions.

The arbovirus of paramount significance concerning central nervous system (CNS) complications is the West Nile virus (WNV) (Miller 2008). Most symptomatic instances of WNV present with mild, influenza-like symptoms; however, less than 1% of infected individuals progress to severe and potentially fatal forms of encephalitis or meningitis. The WNV frequently precipitates the onset of West Nile neuroinvasive disorders (WNNDs), which encompass conditions such as aseptic meningitis, paralysis, and encephalitis. The mortality rate among WNV patients who experience neurological complications is approximately 10%.

Japanese encephalitis, attributed to the Japanese encephalitis virus (JEV), is prevalent in some of the most densely populated nations- such as China, India, Japan, and the entirety of Southeast Asia. Most individuals infected with JEV exhibit no symptoms, with symptomatic cases manifesting in less than 1% of the infected population. Nonetheless, approximately 25% of those who progress to encephalitis succumb to the illness, and among those who survive, 30–50% experience psychiatric, neurologic, or cognitive deficits (CDC 2019). St. Louis encephalitis (SLE), resulting from the St. Louis encephalitis virus (SLEV), represents a rare variant of encephalitis, with symptoms appearing in fewer than 1% of individuals infected. While many patients remain asymptomatic, a limited number exhibit symptoms that can vary from mild, flu-like manifestations to severe encephalitis. The mortality rate among symptomatic patients ranges from 5% to 15% (CDC 2019).

The pathogenesis of the virus is contingent upon the replication of the virus within the lymph nodes and skin keratinocytes of the host (Lim et al. 2011a), culminating in primary viremia. Subsequently, the virus can disseminate to secondary sites, including the central nervous system (CNS), following its traversal across the blood-brain barrier (BBB) (Lim et al. 2011b). The invasion of the CNS by West Nile Virus (WNV) activates Toll-like receptor 3, which elevates the levels of tumour necrosis factor-alpha (TNF-α) and other pro-inflammatory mediators, thereby augmenting the permeability of the BBB and precipitating neuronal death (Lim et al. 2011b). Consequently, arboviral infections can instigate the emergence of various neurological complications, encompassing meningitis. Viral meningitis symptoms can be similar to bacterial meningitis, so it is essential to seek urgent medical help.

8.9.2 Enteroviruses (EVs)

Enteroviruses (or non-polio enteroviruses, coxsackieviruses, or echoviruses) are the most common causative agents of viral infection of CNS (Chen et al. 2007; Frantzidou et al. 2008). Enteroviral meningitis's most common severe complications are meningoencephalitis, myocarditis, and pericarditis. In children, neurologic complications of enteroviral infection can include acute flaccid paralysis and rhombencephalitis. The intestinal mucosa of the gastrointestinal tract (GIT) and the epithelial cells of the respiratory tract (especially the nasopharynx and oropharyngeal) are the two main sites where enteroviruses replicate once they enter the body (Majer et al. 2020). They traverse the intestinal lining and arrive at Peyer's patches, where they continue to multiply after attaching to specific receptors on the surface of enterocytes in the lower GIT (Rotbart 2000).

Studies have also demonstrated that EVs target and penetrate various neural cell types, including glial cells and astrocytes (Majer et al. 2020). For instance, strains of EV-D68 demonstrated the capacity to infect and propagate within a human neuroblastoma cell line and in a postnatal cortical neuron culture (Brown et al. 2018). Furthermore, the virus can access the bloodstream via the infection of immune cells, potentially resulting in secondary infections at various anatomical sites, including the heart and central nervous system (Palacios and Oberste 2005). The dissemination of this virus consequently leads to an array of complications, including meningitis and encephalitis (de Crom et al. 2016). The virus replicates and then hematogenously traverses to the organs it attacks. Enteroviruses enter the bloodstream and reproduce outside of the central nervous system (CNS), where they enter through the blood.

Furthermore, the virus can infect immune cells to enter the bloodstream, leading to secondary infections in various places, such as the heart and central nervous system (Palacios and Oberste 2005). Moreover, enteroviruses can spread vertically from an infected mother to her child by breastfeeding or placenta crossing (Méreaux et al. 2017). Meningitis and encephalitis are two consequences that may result from this diffusion (de Crom et al. 2016). These viruses can commonly cause different

clinical outcomes such as colds, sore throats, stomach upsets, diarrhoea, respiratory illness, hepatitis, pancreatitis, myocarditis, and hand, foot, and mouth (HFM) disease (Akuzawa et al. 2014; Wells and Coyne 2019). Enterovirus meningitis typically has a benign course, while enterovirus encephalitis can result in long-term neurological sequelae. Significant morbidity and mortality follow enteroviral meningitis in neonates and immunocompromised patients.

Echoviruses (EV), subtypes such as EV-6, EV-9, EV-11, EV-13, EV-19, and EV-30, belong to the Enterovirus B species and are frequently linked to cases of viral meningitis, particularly in children. These echovirus strains are well-recognized causative agents of viral meningitis and pediatric infections of the CNS (Kim et al. 2012). The EV30 influences the triple functional domain (TRIO) protein, which is integral to the processes of neuronal cell migration and axonal guidance through a GEFD1-dependent mechanism. EV30 also impairs the functionality of guanine exchange factor domain 2 (GEFD2), culminating in the activation of Ras homology family member A (RhoA), a GTPase involved in signalling pathways. This activation subsequently triggers both the ROCK and MLC signalling cascades. The resultant sequence of events facilitates actin polymerization and increases nitric oxide levels, culminating in neuronal injury and complications within the central nervous system (Lee et al. 2012; Kohil et al. 2021).

Another Enterovirus B species, Coxsackievirus B, is recognized for its pathological role in meningitis and various neurological pathologies (Berger et al. 2006). The mechanism for coxsackievirus B entry into the CNS involves the translocation of infected mononuclear cells (Mac3) that possess the ability to pass the BBB and infiltrate the choroid plexus epithelium, thereby facilitating viral dissemination within the CNS (Tabor-Godwin et al. 2010; Huang and Shih 2015). This process culminates in the activation of extracellular-signal-regulated kinases-1 and -2 (ERK-1/2), subsequently enhancing viral replication within leukocytes, mostly T-lymphocytes (Opavsky et al. 2002). Additional research has indicated that coxsackievirus B replicates within monocytes, B-cells, and T-cells during the viremia phase, with the susceptibility of immune cells to viral infection influencing the extent of secondary organ injury (Hwang et al. 2012). Such manifestation causes severe meningitis following entry into the CNS. Furthermore, histopathological examination of cerebral tissues from mice whose mothers were inoculated with coxsackievirus B4 revealed indications of meningitis, accompanied by the accumulation of immune cells (mainly T-cells) and elevated production of pro-inflammatory cytokines and chemokines such as IL6, TNF-α, IFNα, and MCP-1 (Jmii et al. 2020).

Enterovirus D68 (EV-D68) is the most prevalent member of the Enterovirus D species associated with aseptic meningitis. It is also linked to neurological complications such as meningitis and flaccid paralysis in children (González-Sanz et al. 2019). Unlike other enteroviruses, EV-D68 is primarily a respiratory virus, entering the body through the upper respiratory tract (URT) by binding to the N-acetylneuraminic acid (NA) α2,6-galactose receptor on epithelial cells, which is abundant in the URT (Esposito et al. 2015). This receptor specificity may limit the virus's spread and explain why progression to neurological complications is rare. EV-D68 can also bind to ICAM-5-telencephalin, a receptor found on dendrites and

neurons, facilitating its entry into nerve cells (Wei et al. 2016; Sun et al. 2019). Additionally, EV-D68 may access the CNS via receptors on the olfactory nerve in the nasal cavity (Messacar et al. 2018). These pathways highlight EV-D68's potential involvement in neuropathogenesis and its link to aseptic meningitis.

8.9.3 Human Parechovirus (HPeV)

This family of viruses is closely related to enteroviruses, with a similar infection mechanism primarily involving oral-faecal transmission. Human parechovirus type 3 (HPeV-3) is increasingly recognized as a causative agent of neonatal meningoencephalitis and sepsis (Wolthers et al. 2008). HPeV can be spread between individuals through respiratory secretions and saliva, often leading to respiratory infections (Wolthers et al. 2008; Johannessen and Burns 2012; González-Sanz et al. 2019). However, it can also cause neurological conditions like meningitis and encephalitis, especially in children (Wolthers et al. 2008). Unlike other HPeV serotypes that use the arginine-glycine-glutamic acid (RGE) sequence motif at the carboxyl terminus of the capsid protein (VP) for receptor binding, HPeV-3 uses a different receptor binding site (RBS) (Westerhuis 2015). So far, no specific receptor has been identified for HPeV-3, which could explain its unique tropism (Karelehto et al. 2018). The absence of a receptor in the respiratory tract might also account for why HPeV-3 often leads to meningitis and neonatal sepsis, whereas HPeV-1 is more associated with respiratory and gastrointestinal illnesses.

8.9.4 Human Herpesvirus

Herpesviruses from the family Herpesviridae, including HSV1, HSV2, VZV, EBV, and HHV-6, are known to cause meningitis and can establish latent infections in neurons with potential for reactivation (Spear and Longnecker 2003). HSV infections are associated with encephalitis and meningitis, often leading to significant neurological consequences (Whitley and Lakeman 1995). Especially HSV2 has been linked to Recurrent Lymphocytic Meningitis (RLM), or Mollaret's meningitis, a rare form of viral meningitis characterized by sudden episodes of symptoms lasting 2–7 days, with symptom-free intervals ranging from weeks to years. Viral entry of HSV1 and HSV2 involves four key glycoproteins (gB, gD, gH, and gL), with cell binding primarily mediated by gB or gC through interaction with cell-surface heparan sulfate (Spear and Longnecker 2003). Three known receptors for HSV include the herpesvirus entry mediator (HVEM), members of the immunoglobulin (Ig) superfamily, and heparan sulfate (HS). The HVEM receptor is found on various cells, such as oropharyngeal epithelial cells and neurons, facilitating viral entry and possibly contributing to meningitis. EBV infection begins in oropharyngeal epithelial cells and spreads to lymphoid tissue, primarily targeting B-cells where it can

establish latent or, less commonly, lytic infections. During latency, EBV expresses Epstein-Barr nuclear antigens (EBNAs) and latent membrane proteins (LMPs) (Akkoc et al. 2016). EBV can also infect brain endothelial cells, leading to latent infection. Stress in the brain can reactivate the virus, triggering an inflammatory response through increased expression of cytokines and chemokines like IL-1, IL-12, IL-18, and TNF-α (Koyuncu et al. 2013).

8.9.5 Orthomyxoviruses

Human influenza viruses, members of the Orthomyxoviridae family, are primarily known for causing pneumonia and acute respiratory distress syndrome (ARDS) (Liang et al. 2018). However, in rare instances, these viruses can also lead to extra-respiratory diseases, including encephalitis (Sejvar and Uyeki 2010), meningoencephalitis, myelitis (Xia et al. 2014), and meningitis (Liang et al. 2018). Research suggests that influenza viruses may access the CNS through the olfactory route (Yamada et al. 2012) and other cranial nerve cells (Siegers et al. 2019). The virus typically binds to α-2,3-linked sialic acid and α-2,6-linked sialic acid receptors present on the epithelial cells of the upper respiratory tract (URT) and lower respiratory tract (LRT) (Imai and Kawaoka 2012). Notably, these sialic acid receptors are also expressed in neurons within the cerebral-cortex and brain-stem (Siegers et al. 2019), which may facilitate viral spread to the CNS and explain its association with neurological syndromes.

8.9.6 Paramyxoviruses

Before the widespread use of the measles-mumps-rubella (MMR) vaccine, mumps virus (MuV) was a leading cause of viral meningitis in children, particularly in developed countries (Rotbart 2000; Sawyer 2002). Despite vaccination efforts, recent years have seen multiple mumps outbreaks in developed regions, including the USA (Marlow et al. 2020; Su et al. 2020). Mumps virus, a member of the Paramyxoviridae family, can lead to severe complications such as encephalitis, meningitis, orchitis, myocarditis, pancreatitis, and nephritis (Betáková et al. 2013; Rubin et al. 2016). MuV is transmitted via respiratory or oral routes through contact with infected respiratory droplets or secretions, with an incubation period ranging from 2 to 4 weeks (Maillet et al. 2015). The virus binds to sialic acid to enter polarized epithelial cells in the upper respiratory tract, facilitating further invasion into adjacent cells (Katoh et al. 2015). Approximately half of MuV infections are asymptomatic or present only mild respiratory symptoms or fever. The virus is highly neurotropic and can directly infect the central nervous system (Bockelman et al. 2018), and due to high viremia, it can also disseminate to other organs such as the heart, kidneys, and genital organs (Ternavasio-de la Vega et al. 2010). MuV efficiently infects T-cells,

allowing mature viruses to migrate and disseminate to various organs, which plays a crucial role in the pathogenesis and spread of mumps disease.

8.10 Molecular Diagnosis and Detection of Viral Meningitis

Multiple types of pathogens like bacteria, fungi, and viruses can enter, invade, and infect the membranes that line the brain and spinal cord, causing syndromic meningitis, and the condition can easily become fatal if not diagnosed in time and left untreated. Successful clinical outcomes for patients with viral meningitis are clearly associated with confirmation of diagnosis. Hence, we need accurate, fast, sensitive, and precise tests to identify the causative viral pathogen for appropriate clinical treatment of meningitis (Diallo et al. 2021). During viral meningitis, the viruses enter the CNS primarily by three routes—axonal transport, by penetrating the blood-brain barrier and entry mediated by the Trojan Horse method. All three entry routes leave distinguishable traces that can be detected by employing different diagnosis methods (Cain et al. 2019).

Meningitis caused by enteroviruses may also be hard to differentiate from semi-treated bacterial meningitis, as CSF pleocytosis tends to have early neutrophil predominance. However, there are multiple challenges to enterovirus diagnosis, such as the high rate of mutation of these viruses, increased shedding times of enteroviruses and certain strains, such as EV-71, being difficult to detect in the cerebrospinal fluid. Diagnosis of CNS infections also becomes difficult due to challenges in sampling the site of infection via brain biopsy or lumbar puncture; thus, the sample collected is often limited. All these challenges can be overcome by using molecular approaches to diagnosis, such as nucleic acid amplification techniques (Ex-PCR), which test for enterovirus RNA in blood (Sawyer 2001).

8.10.1 Traditional Diagnostic Methods

Non-molecular methods used clinically to diagnose viral meningitis are isolation and culture of the virus, microscopic examination of the sample, serological studies including ELISA and immune-based detection of viral or viral antigens, including immunofluorescence.

8.10.2 Microscopic Examination

The microscopic examination uses electron microscopy to determine the viral pathogen, but it does not have specificity for a particular group of viruses and has an exceptionally high detection limit (10^6–10^7 particles/mL); thus, viruses in the CSF need to be concentrated (Dronina et al. 2021).

8.10.3 Virus Isolation

Virus isolation and culture have been considered the gold standard for diagnosis since the 1900s. Enteroviruses and herpes simplex virus are isolated from the patient's cerebrospinal fluid. However, the limits of this technique are that it is time-consuming (3–7 days), it shows low sensitivity (65–75%) and has high cost and, hence, has minimal clinical utility nowadays (Kadambari et al. 2014).

8.10.4 Immunofluorescence Detection

This test uses fluorescence microscopy to detect viral antigens or antibodies in the CSF sample. It can detect virus location within the cells and expression of viral proteins, but it can only be used when infected cells are absent (Gagliardi et al. 2022).

8.10.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA uses antigen-antibody specificity to detect viral samples in the CSF. Using ELISA to detect enterovirus-specific IgM in meningitis patients is more rapid, sensitive, and cost-effective than virus isolation (Ito et al. 2021). ELISA developed for detecting SARS-CoV-2 IgM showed a sensitivity of 85% compared to RT-PCR and a specificity of 100% (Cusi and Savellini 2011). The diagnosis of arboviral encephalitis is primarily based on clinical presentation and serological testing of serum or cerebrospinal fluid (CSF). However, the U.S. Food and Drug Administration (FDA) has approved specific diagnostic tools, including the Zika MAC-ELISA and Zika virus RNA Trioplex RT-PCR assay, which detect the virus in blood and urine samples, confirming the infection.

8.11 Molecular Diagnostic Methods

Molecular methods of detection of viral meningitis, especially polymerase chain reaction, are now becoming commonplace in clinical settings and are increasingly becoming the new gold standard for the detection of the aetiology of viral meningitis as they exhibit a greater rate of detection without affecting viral viability (Cusi and Savellini 2011). Virus isolation from CSF was previously the established standard for viral pathogens causing meningitis, but as the process was slow, cost-prohibitive, and exhibited low sensitivity, molecular approaches to diagnosis are replacing it. In a study, the detection rate was increased from 8.9% to 14.8% using real-time PCR assays and reverse transcription methods of diagnosis. The sensitivity, cost, speed, and specificity of molecular diagnostic tests for meningitis have seen rapid improvements in the past decade (Dupuis et al. 2011).

8.11.1 PCR-based Nucleic Acid Detection and Quantification

Nucleic acid detection of viral meningitis is done mainly using PCR, which is used for multiplex replication of specific DNA fragments in a laboratory setting, resulting in the exponential amplification of the DNA sequence of interest. Nucleic acid amplification from CSF samples is used primarily during the acute phase of infection (Sanbonmatsu-Gámez et al. 2021). PCR is now-a-days routinely and widely used in clinical practice as it reduces the time of diagnosis, does not affect viral viability, reduces the risk of contamination in pathogen detection and has high sensitivity. PCR has high sensitivity and specificity and a low turnaround time of about 1–2 h. Also, because of the rapidity of this method, it is not affected by virus viability in specimens. We can use multiplex PCR to combine different targets in a single PCR reaction, making diagnosis less time-consuming. Rapid multiplex molecular diagnostic tests for various CNS infections can reduce hospitalization and antibiotic treatment duration (Weinberg et al. 2002).

Real-time PCR, also known as quantitative PCR (qPCR), is used to study the amplification of target DNA sequences in real-time by fluorescence probing and monitoring fluorescence signal accumulation. This has been increasingly used in clinical diagnosis because of its simplicity, accessibility, specificity, and sensitivity (Khumalo et al. 2017).

Reverse Transcriptase PCR is another molecular diagnostic approach to detect RNA expression by creating complementary DNA (cDNA) from RNA. It can be used to study gene expression and quantitative detection of RNA. It has been used for detecting non-polio enteroviruses (NPEVs), human parechovirus (HPeV), and the Toscana Virus. This technique offers high sensitivity, rapidity, and reproducibility (Gupta et al. 2022).

Multiplex primer PCR is now the most commonplace nucleic acid detection technique, but the diagnosis through PCR is only possible if the infectious agent is known. Thus, the challenges are the high cost of multiplex PCR and the limited range of known targeted pathogens (Oechslin et al. 2018).

Microarray assays can also rapidly detect pathogen DNA in which species-specific oligonucleotide probes are fixed onto wells and detected using fluorescent dye hybridization (Hou et al. 2018).

8.11.2 Metagenomic Next Generation Sequencing (mNGS)

Metagenomic Next Generation Sequencing (mNGS) is a novel and upcoming method that simultaneously identifies all different types of pathogens (viral, bacterial, fungal) in a human sample. In metagenomic sequencing, a complementary DNA strand is generated from every fragment of genomic material in our sample, and bioinformatic analysis is performed to remove human sequences. Finally, the remaining fragments are assembled in comparison to known sequence databases. Novel pathogens can thus be identified using partial matches to conserved regions of known sequences (Gu et al. 2019).

It has been found to identify viruses that are generally not detected by nucleic acid testing. A systematic review of metagenomics studies for diagnosing patients with test-negative encephalitis identified 44 cases in which 18 novel, 5 rare, and 5 unexpected pathogens (bacterial, fungal, and viral) were found. This illustrates the advantage of using metagenomic sequencing for diagnosis compared to targeted molecular methods (Wilson et al. 2018).

8.11.3 Targeted Next-Generation Sequencing (tNGS)

In targeted next-generation sequencing, specific sequences are sequenced with the help of polymerase chain reaction or probe hybridization. It has successfully identified the herpes virus, all human enterovirus serotypes and five flaviviruses (Korimbocus et al. 2005).

Results obtained by sequencing can be affected by contamination, collation, low concentration of pathogens and the range of databases used as reference for analysis of bioinformatics. Thus, PCR and sequencing are often used in conjunction with each other's technique (Miller et al. 2019).

If Viral PCR is negative for aseptic meningitis, then serologic testing along with Next Generation Sequencing can be a meaningful method for diagnosis. Thus, NGS of CSF combined with routine tests will have the highest diagnostic efficiency (Wilson et al. 2019).

8.12 Meningitis Disease control, Prevention, and Therapeutics

Currently, no specific antiviral treatments are available for arboviral diseases; thus, management is limited to supportive care and symptomatic relief. For Zika virus infection, diagnosis traditionally relies on clinical symptoms. No antiviral treatments or vaccines are available for the Zika virus, so management remains supportive. Mostly, enteroviral meningitis resolves itself on its own and needs supportive care. Similarly, HSV meningitis is self-limiting in immunocompetent patients, and symptomatic treatment alone is usually sufficient. Moreover, a 7–10 days course of antiviral medication with acyclovir, valacyclovir, or famciclovir, however, has been demonstrated to enhance neurological results in immunocompromised persons (Noska et al. 2015). It is crucial to point out that HSV meningitis, which HSV-2 usually causes, is not the same as HSV encephalitis, which is caused by HSV-1 (Logan and MacMahon 2008). Treatment with IV acyclovir is necessary to prevent fatalities as well as disabilities in HSV encephalitis caused by HSV-2. Although the optimal course of treatment for HSV-1 or VZV meningitis is still unknown, many medical professionals would give antivirals unless the patient was already

improving (Logan and MacMahon 2008). Meningitis brought on by an acute HIV infection requires ongoing monitoring and care from an experienced physician.

Vaccination prevents viral infections that can lead to encephalitis and meningitis. Vaccination against herpes zoster virus (HZV) with vaccines such as Shingrix (recombinant zoster vaccine) and Zostavax is recommended for adults over 50 years old to prevent herpes zoster (shingles), a potential cause of viral meningitis (US-CDC 2024). These vaccines are crucial in reducing the incidence and severity of viral infections that can lead to debilitating neurological conditions like encephalitis and meningitis.

Three major classes of meningococcal vaccines are available to prevent meningococcal disease. The conjugate vaccines known as MenACWY, which include Menveo® and MenQuadfi®, are intended to protect against meningococcal serogroups A, C, W, and Y. Adolescents, college students living in dorms, HIV-infected, prospective military personnel, and travellers to regions like the meningitis belt in sub-Saharan Africa are all advised to get these vaccinations (US-DHHS 2019). Second, the MenB vaccines target meningococcal serogroup B using recombinant protein technology. Examples of these vaccines are Bexsero® and Trumenba®. For people who are more likely to encounter serogroup B strains—such as college students in areas where outbreaks are occurring—these vaccinations are incredibly crucial. Third, the MenABCWY vaccine PenbrayaTM offers comprehensive protection against serogroups A, B, and C by combining conjugate and recombinant protein technology. Based on risk assessment and public health guidelines, this vaccine may be administered to populations and provides comprehensive protection.

Dexamethasone (an anti-inflammatory drug) plus ceftriaxone (antibiotics) is the first-line empirical treatment for individuals with suspected bacterial meningitis (BM). Dexamethasone decreases pro-inflammatory cytokines (IL-1β and TNF-α in CSF) and inhibits leukocytes' reactive oxygen species (ROS). Vancomycin should be added if the risk of pneumococcal meningitis is increased or if there has been recent usage of β-lactam antibiotics. Benzylpenicillin should be added if there are risk factors for Listeria meningitis present. When treating bacterial meningitis, dexamethasone helps lower intracranial pressure (ICP), oedema, and cerebral inflammation (Borchorst and Møller 2012). It should be given either prior to or concurrently with the initial antibiotic dose to reduce cerebral inflammation brought on by bacteriolysis generated by antibiotics. If cerebrospinal fluid (CSF) characteristics are consistent with viral meningitis and there is minimal clinical suspicion of bacterial meningitis, both dexamethasone and antibiotics can be stopped.

8.13 Challenges in Molecular Diagnosis of Viral Meningitis

Meningitis results in several challenges, individually, medically, and socially. Individually, it can cause brain damage, hearing loss, or learning disabilities in 10–20% of survivors (WHO 2023). Additionally, it can cause memory loss, lack of concentration, difficulty retaining information, clumsiness, and coordination

problems (Meningitis Research Foundation 2024). In some cases, meningitis can lead to death, with approximately 300,000 deaths occurring worldwide each year (Barichello et al. 2023). Furthermore, meningitis can cause significant cognitive and physical impairments, affecting daily life and overall well-being. Medically and socially, there are many challenges, some of which are mentioned below.

8.13.1 Diagnostic Challenges

Diagnosing meningitis can be challenging due to its nonspecific symptoms, similar to other conditions. The differential diagnosis of meningitis includes stroke, subdural hematoma, subarachnoid haemorrhage, metastatic brain disease, and brain abscess (Hersi et al. 2024). Additionally, patients may present with a range of symptoms, including neck stiffness, confusion, coma, and cranial nerve palsy, making it difficult to pinpoint the exact diagnosis (Foppiano Palacios and Saleeb 2020). Furthermore, diagnosing meningitis relies on a combination of medical history, physical examination, and laboratory tests, which can be time-consuming and may not always yield conclusive results. Evaluating cerebrospinal fluid is crucial in diagnosing meningitis, but even this may not be straightforward (Bamberger 2010). Moreover, the diagnosis can be complicated by "partially treated meningitis", where patients exhibit meningitis symptoms after receiving antibiotics for another condition. Diagnosing meningitis requires a high index of suspicion and careful evaluation to ensure prompt and accurate diagnosis.

8.13.2 Fluidity of Infectious Agents

The changing pathogen landscape concerning meningitis poses several challenges, including the shift of invasive infection and risk factors, as well as the need to adapt to changing epidemiology due to factors such as antibiotics, vaccination, and travel patterns (Allonce et al. 2024; Skar et al. 2024). Additionally, the diverse range of viruses that can cause meningitis, with varying geographical and seasonal distributions, affects people of different ages, making it a complex issue to address (Kwambana-Adams 2023). Furthermore, the molecular and bioinformatics challenges associated with bacterial meningitis must also be considered (Ramachandran and Wilson 2020).

8.13.3 Vaccination Programs

Vaccination against meningitis faces several challenges, including exposure to disease-causing bacteria and improper carbohydrate-protein conjugations in current vaccines (Bagwe et al. 2020). Additionally, despite advances in vaccine

development, bacterial meningitis and sepsis remain significant public health challenges globally (Alderson et al. 2021). Furthermore, there is no universal vaccine against meningococcal disease, and vaccine hesitancy is a significant obstacle to widespread vaccination.

8.13.4 Resistance against Antibiotics

Antibiotic resistance poses significant challenges in the treatment of meningitis, including the inability of antibiotics to pass the BBB and the increasing resistance of causative agents, leading to poor treatment outcomes and increased antibiotic resistance (Kietzman and Tuomanen 2019; Ali et al. 2021). The use of antibiotics in areas with resistance can result in increased suffering and death. Furthermore, infections caused by resistant bacteria lead to higher rates of adverse outcomes than susceptible strains (Friedman et al. 2016). The implications of antibiotic resistance in meningitis treatment are severe, including increased mortality, neurological damage, and long-term after-effects such as hearing loss, seizures, and cognitive impairments (WHO 2023). To prevent these outcomes, it is essential to initiate prompt and appropriate antimicrobial therapy.

8.13.5 Fast tourism and Globalization

Travel and globalization have significantly contributed to the changing pathogen landscape and epidemiology of meningitis. The increased speed, volume, and reach of travel and migration have facilitated the rapid spread of infectious diseases, including meningitis, across different geographic regions (Wunrow et al. 2023). Globalization has led to the interconnectedness and interdependence of countries, resulting in the exchange of goods, services, and people, which can spread pathogens (Kwambana-Adams 2023). This has altered the distribution of meningitis, making it a global health concern. Furthermore, the interplay of environmental factors, such as climate change, has also impacted the epidemiology of meningitis (Mazamay et al. 2020). The movement of people from one region to another has increased the risk of transmission, and the changing behaviours associated with globalization have also contributed to the spread of the disease. The Global Meningococcal Initiative has highlighted the importance of understanding the global epidemiology of meningococcal disease and the impact of vaccines on its prevention. Overall, the impact of travel and globalization on the changing pathogen landscape and epidemiology of meningitis is complex and multifaceted, requiring a comprehensive approach to address this global health issue. Addressing these challenges requires a multi-pronged approach involving healthcare providers, public health officials, researchers, policymakers, and communities working together to improve prevention, diagnosis, treatment, and support for those affected by meningitis.

8.14 Conclusion and Future Scope

Bacterial meningitis caused by *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* remains a severe threat because of its high mortality rate and prolonged neurological sequelae. Viral meningitis, caused by arboviruses, enteroviruses, and herpesviruses, is more prevalent and less severe. This chapter focuses on understanding bacterial and viral meningitis's pathogenesis, epidemiology, and diagnostics. These bacterial and viral meningitis have many common clinical presentations. Therefore, early and differential diagnosis is essential for effective treatment. Molecular diagnostics, notably PCR-based approaches and NGS, have revolutionized meningitis diagnosis by quickly, accurately, and precisely identifying the causative viral and bacterial agents. Despite these advances in identifying viral and bacterial meningitis, more targeted treatments, especially those against antibiotic resistance, are the need of the hour for reassessment.

Immunisation has been demonstrated to reduce bacterial meningitis. Especially meningococcus, pneumococcus, and H. influenzae type b vaccines have significantly decreased these diseases. However, non-vaccine serotypes and vaccination gaps highlight the need for broader-spectrum vaccine research and development. Metagenomic sequencing and point-of-care PCR will enhance early and pathogen differential detection. Novel anti-inflammatory, neuroprotective and targeted antibacterial therapies with the least side effects will reduce inflammation and neurological damage. Meningitis may be decreased by improving vaccination coverage and developing vaccines against non-bacterial pathogens such as enteroviruses and arboviruses across all their serotypes. To overcome antibiotic resistance, novel drugs and therapies are needed to treat bacterial meningitis.

In conclusion, meningitis diagnostics and treatments have advanced, but many unanswered questions still need more research. Future efforts should centre on vaccination coverage, innovative treatment approaches, and diagnostic precision to lessen the worldwide impact of this awful illness. Improved healthcare infrastructure, public awareness of meningitis, and worldwide monitoring systems might reduce morbidity and mortality.

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Chapter 9
Molecular Diagnosis of Human Papillomavirus: Current Trends and Recent Approaches in Molecular Diagnosis

Saif Khan, Md Shahadab, Aditya Goel, Aman Haidar Rizvi, Mohammad Akram, Md. Kausar Neyaz, Manu Sharma, and Mairaj Ahmed Ansari

Abstract Human papillomavirus (HPV), a major etiological factor of cervical cancer and other anogenital malignancies, having more than 200 genotypes divided into five genera alpha(α), beta(β), gamma(γ), mupa, and nupa, out of which 40 are associated with cancer and other malignancies. Traditional methods of diagnosis such as cytology and histopathology show compromised sensitivity and specificity, leading to a growing reliance on molecular techniques. Molecular diagnosis of HPV plays a crucial role in early detection, genotyping, and disease management. Recent advancements Polymerase-chain-reaction (PCR)-based and CRISPR-based diagnostics assays offer high sensitivity and specificity for HPV detection and genotyping with improved accuracy, rapid results, and cost-effective screening. Additionally, isothermal amplification methods such as loop-mediated-isothermal-amplification (LAMP) have emerged as promising point-of-care diagnostic tools. The integration of biosensors, microfluidics, and artificial intelligence in HPV diagnostics is further

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enhancing precision and accessibility. Despite these advancements, challenges such as cost, accessibility in low-resource settings, and the need for standardized protocols still exist. In this chapter, we highlight the current trends and recent approaches in molecular diagnosis of HPV, and their potential utilization in clinical practices. The development of rapid, accurate, and affordable molecular diagnostic tools will improve HPV screening programs and reducing the global burden of HPV-associated diseases.

Keywords Human Papillomavirus (HPV) · Molecular diagnosis · Real-Time PCR (qPCR) · HPV genotyping · CRISPR-based diagnostics · Loop-Mediated Isothermal Amplification (LAMP) · High-risk HPV strains · HPV associated cervical cancer · Screening and early detection · Challenges in HPV molecular testing

9.1 Introduction

Human papillomaviruses (HPVs) are 55 nm size viruses, non-enveloped, having circular, double-stranded 7900 bp length DNA genomes. Its icosahedral capsid is constructed from 72 capsomers, housing at least two capsid proteins major L1 and minor L2. L1, the major capsid protein, forms each capsomer as a pentamer (8 in total) around 12 copies of L2 are involved in each capsid. Classified within the *Papillomaviridae* family, they demonstrate extensive genetic diversity, comprising over 200 identified types organized into 39 genera (Wang et al. 2018).

The diverse and complex genome of HPV is structured into three main segments (i) the upstream regulatory region (URR), (ii) the first area, and the second area. (iii) The URR also referred to as the long control region (LCR) or non-coding region (NCR), constitutes around 10% of the genome and serves as a regulatory centre for viral transcription, replication, and genome maintenance. The first area, representing nearly half of the genome, is divided into two large reading frames, E1–E2 and E4–E7, along with numerous small reading frames. Notably, the E6 and E7 genes in the small reading frames encode oncoproteins pivotal in transforming infected cells and contributing to the oncogenic potential of high-risk HPV. The second area constitutes the remaining 40% of the genome and harbors the genes L1 and L2. These genes encode structural proteins crucial for forming viral capsids during the later stages of the viral life cycle. The HPV gene and its activities are mentioned in Fig. 9.1.

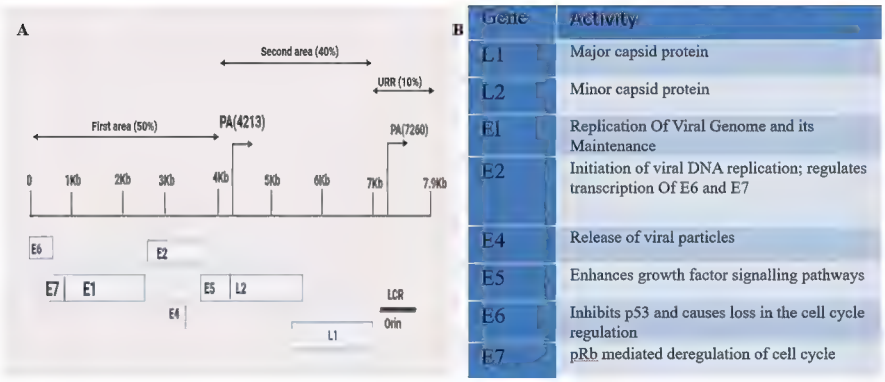

Fig. 9.1 The schematic diagram for linear HPV genome structure (**a**). The functions of early (E) and late (L) genes (**b**)

9.2 HPV Classification

Each HPV type is genetically distinct, with a classification requiring a 10% difference in the L1 nucleotide sequence from other types (Levakov et al. 2022). Types are numbered chronologically by discovery date (Wang et al. 2018).

Out of 39 genera in the Papillomaviridae family, five are known as HPVs and infect human: Alphapapillomaviruses (14 species and 65 HPV stains or genotypes), Betapapillomaviruses (6 species and 52 HPV stains or genotypes), Gammapapillomaviruses (27 species and 81 HPV stains or genotypes), Mupapillomaviruses (3 species and 3 HPV stains or genotypes), and Nupapapillomaviruses (1 species and 1 HPV stains or genotype), (De Villiers et al. 2004; Yang et al. 2022). In 2012, the International Agency for Research on Cancer (IARC) classified HPVs into groups based on their carcinogenicity as shown in Fig. 9.2.

Group 1 (Carcinogenic to humans): HPV 51(sp. Alpha 5), 56(sp. Alpha 6), 18(sp. Alpha 7), 39(sp. Alpha 7), 45(sp. Alpha 7), 59(sp. Alpha 7), 16(sp. Alpha 9), 31(sp. Alpha 9), 33(sp.
Alpha 9), 35(sp. Alpha 9), 52(sp. Alpha 9) and 58(sp. Alpha 9)

Group 2A (Probably carcinogenic to humans): HPV 68(sp. Alpha 7)

Group 2B (Possibly carcinogenic to humans): HPV 26(sp. Alpha 5), 69(sp. Alpha 5),82(sp. Alpha 5), 30(sp. Alpha 6), 53(sp. Alpha 6), 66(sp. Alpha 6), 70(sp. Alpha 7), 85(sp. Alpha 7),
97(sp. Alpha 7), 67(sp. Alpha 9), 34(sp. Alpha 11) and 73(sp. Alpha 11).

96% of cervical cancers are linked to HPV types in groups 1 and 2A. Although group 2B HPVs are less common in cervical cancer cases, they show markers of carcinogenesis, such as E6 mRNA, increased p16 expression, and decreased cyclin D1, p53, and Rb expression (Wang et al. 2018).

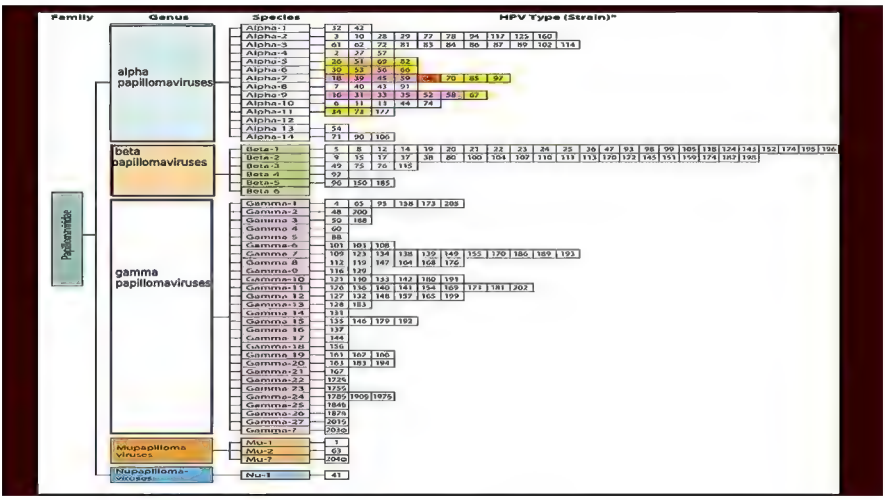

Fig. 9.2 Classification of Human Papillomavirus (Burd 2016) Pink = IARC group 1 (carcinogenic to humans) carcinogen, Orange = IARC group 2A (probably carcinogenic to humans) carcinogen Yellow = IARC group 2B (possibly carcinogenic to humans) carcinogen Species Alpha-12 and Beta-6 are animal papillomaviruses. HPV types 46, 55, 64, and 79 have been reclassified, leaving their numbers vacant. ΦHPV type 203 has been classified as a gammapapillomavirus and HPV 204 as a mupapillomavirus but species designations have not yet been made

9.3 HPV Proteins in Cancer Pathogenesis and Disease Progression

The HPV virus possesses an icosahedral capsid that homes 8 overlapping open analyzing frames that incorporate early (E) and overdue (L) genes in addition to an untranslated prolonged regulatory region that plays an important role in pathogenesis. The major and minor capsid proteins are encoded with the aid of using the L1 and L2 genes. About 12 molecules of L2 and 72 pentamers of L1 are gifted with inside the capsid (Handisurya et al. 2009).

Infection with the aid of papillomaviruses calls for virus debris benefit to get entry to the epithelial basal layer and input the dividing basal cells. Having entered the epithelial tissues, the HPV virus enters the nucleus of a basal epithelial mobileular, wherein early genes E1 and E2 are expressed, replicating the viral genome and transcribing messenger RNA wanted for viral replication; similarly to its function in replication and genome segregation, E2 also can act as a transcription component and might alter the viral early promoter and manage the expression of the viral oncogenes (E6 and E7). At low tiers, E2 acts as a transcriptional activator, while at excessive tiers E2 represses oncogene expression. As the host cells differentiate, genes E4 and E5 help in the manufacturing of the viral genome with the aid of controlling the epidermal increase component. E6 and E7 are viral oncogenes which now turn out to be important. E6 reasons degradation of the tumor suppressor gene

p53, simultaneously as E7 completes for retinoblastoma protein (pRb), permitting the transcription component E2F to pressure mobileular proliferation processes. The p16 protein, encoded with the aid of using the suppressor gene CDKN2A (MTS1, INK4A) at chromosome 9p21, is an inhibitor of cyclin- based kinases (CDK) which slows the mobileular cycle with the aid of using inactivating the characteristic of the complex-cdk4 and cdk6- cyclin D. These complexes alter the managing factor of the G1 segment of the mobileular cycle with next phosphorylation and inactivation of retinoblastoma (pRb), which E2F launched and which lets in cells to go into S segment. It has been verified that there is a correlation between pRb and p16 reciprocal, which is why there is a robust overexpression of p16 each in carcinomas as in lesions of the premalignant cervix. In cervical cancer, pRb is functionally inactivated from the preliminary tiers of cervical carcinogenesis attributable to the expression of the HPV E7 gene. Genes E6 and E7 consequently act to eliminate precept mechanisms of mobileular defense and pressure the mobileular replication equipment in the direction of the manufacturing of the latest virus debris. E6 and E7 also are recognized to sell oncogenesis. On the other hand, the integration of HPV- DNA into the host DNA is a widely known subject matter in cervical cancer. Integration of HPV sixteen DNA correlates with a disorder of HPV E1 or E2 ORF, which are energetic throughout HPV replication. E2 lack of characteristics leads to the up-law of E6 and E7 oncoproteins because E2 is a repressor of E6 and E7 (Jain et al. 2023) (Fig. 9.3).

The development of cervical cancer is a continuous and gradual process, starts with moderate cervical intraepithelial.neoplasia [CIN1.] and progressing to more severe stages of neoplasia, which include microinvasive.lesions [CIN2. or CIN3], ultimately leading to invasive disease. This trend has served as the basis for secondary preventative measures, therapeutic approaches, and diagnostics. Certain studies

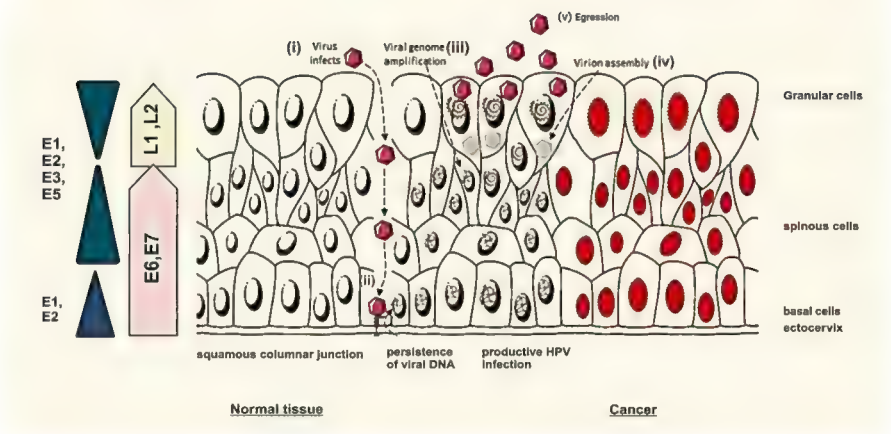

Fig. 9.3 HPV pathogenesis: (i) Interaction of virion with the host, (ii) Entry into epithelial cells, (iii) HPV replication, (iv) L1 and L2 gene expression and capsid formation, and (v) Egression (After Wang et al. 2018)

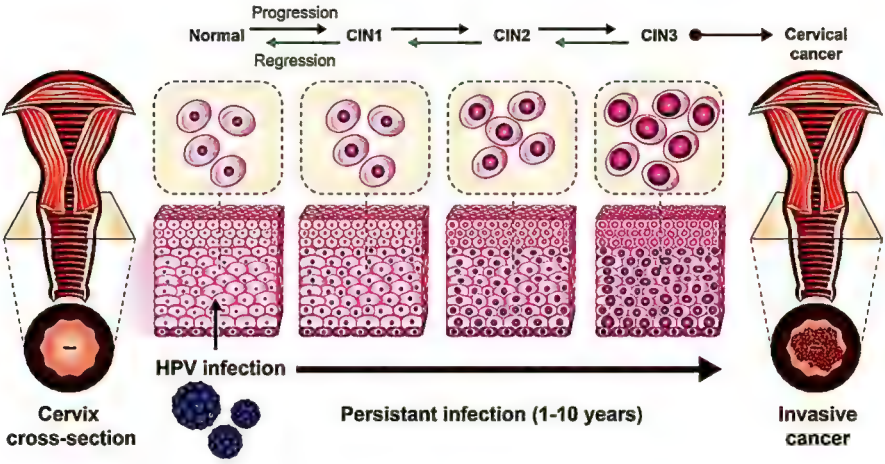

Fig. 9.4 Natural HPV infection is a major cause of cervical cancer. The progression of cervical lesions is categorized using the cervical intraepithelial neoplasia CIN scale: CIN1 represents mild dysplasia, CIN2 indicates moderate dysplasia, and CIN3 encompasses severe dysplasia, including carcinoma in situ

have linked specific HPV types to varying levels of CIN, suggesting that CIN1 represents a self-limiting sexually transmitted HPV infection. However, the actual precursor to cervical cancer is CIN2 or CIN3 as shown in Fig. 9.4 (Yousefi et al. 2022; Stanley 2012).

9.4 Epidemiology

Human Papillomavirus [HPV]. is the most common sexually transmitted viral infection in the world, affecting both men and women. At some point, 85% of women and 95% of sexually active men will contract HPV. This virus is the main cause of nearly all cervical cancer cases, most anal cancers, and a significant number of vaginal, penile, vulvar, and oropharyngeal cancers. In 2020, there were about 604,127 new cervical cancer cases and 341,831 deaths, making it the second most common cancer in women aged 15–44. Infection rates are particularly high in India, Eastern Europe, Latin America, and sub-Saharan Africa, with nearly 90% of cervical cancer deaths occurring in low- and middle-income countries (Publication of IARC Handbooks of Cancer Prevention Volume 18: Cervical Cancer Screening – IARC n.d.).

There are many HPV strains (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) that are classified as carcinogenic, leading to anogenital and oropharyngeal cancers. While most people clear HPV infections within 1 to 2 years, 10–20% of infected women remain persistently infected. Chronic HPV infections,

especially with types 16 and 18, are linked to cervical, anogenital, and head and neck cancers. The burden of HPV-related cancers can be reduced through better HPV vaccination programs and cervical screening based on HPV testing.

Studies show that HPV is most common among healthy women in Sub-Saharan Africa (24%), especially in Eastern Africa (33.6%), and in Latin America. In Asia, regions like Eastern Asia (China) and South-Central Asia (India) have the highest prevalence rates (57.7% and 44.7%, respectively). In contrast, European countries generally report much lower HPV prevalence (<30%), with Western Europe at just 3.7%. Overall, infection rates are higher in developing regions (42.2%) compared to developed regions (22.6%).

HPV infections peak during the teens and twenties and then gradually decline, though some countries see a second peak later in life. The global age-standardized incidence rate (ASIR) of cervical cancer is 13.1 per 100,000 women, with a mortality rate of 6.9 per 100,000. Cervical cancer is the second most common cancer in women under 50 and the fourth most common in women of all ages.

The worldwide prevalence of genital HPV infection in men is comparable to that in women (3.5–45% vs. 2–44%), with analogous transmission rates. Homosexual males and HIV-positive men exhibit a heightened risk, with HPV anal infection rates exceeding 90%, in contrast to heterosexual men, whose risk is proportional to the number of sexual partners. Regional data indicates elevated HPV infection rates among African men, particularly in South Africa (17.2% annually), and diminished rates in Asia (3.2% annually). Research demonstrates a greater incidence of all HPV genotypes in low- and middle-income nations relative to developed ones.

In 2020, India accounted for 24% of HPV-related cancer cases and 7% of all cancer cases worldwide, with four out of five cervical cancers caused by HPV types 16 and 18. As part of cancer prevention in India, health facilities are implementing screenings for common cancers like oral and cervical cancer. Population- and hospital-based cancer registries in India can help track the effects of preventive interventions. Understanding HPV-linked cancer epidemiology is crucial for defining preventive strategies (Human Papillomavirus and Related Diseases Report WORLD n.d.).

9.5 Early Detection

Almost all cervical cancers are caused by HPV infections, but it can take 20 years or more for cervical cancer to develop after an HPV infection. HPV infections and early-stage cervical cancer usually do not produce noticeable symptoms. Getting vaccinated against HPV is the best way to protect yourself from cervical cancer. Since early cervical cancer does not cause symptoms, regular screening tests are crucial for detecting precancerous changes in the cervix that could lead to cancer. Current guidelines recommend that women aged 21–29 have a Pap test every 3 years. For women aged 30–65, it is recommended to continue having a Pap test every 3 years or extend the interval to 5 years if they also undergo the HPV DNA

test. Women over 65 can stop testing if they have had three consecutive normal Pap tests or two HPV DNA and Pap tests with no abnormal results. HPV infections are transient and resolve on their own, persistent infections with high-risk HPV types can lead to precancerous lesions and cancer. Early detection and management of HPV infections are vital for preventing associated morbidity and mortality (PAHO/WHO | Pan American Health Organization n.d.).

Unfortunately, most HPV is detected at a very late stage making the treatment cumbersome. Recognizing the critical role of early diagnosis crucial for shaping effective healthcare policies, public health campaigns, and individual health choices to address the global health impact of HPV comprehensively. The early diagnosis of Human Papillomavirus (HPV) holds significant public health implications, primarily understating cancer progression and in the prevention of cervical cancer, implementation of vaccination. It enables effective management strategies, such as tailored treatments, lifestyle modifications, and counselling, to prevent complications or progression to severe conditions. Ultimately, early diagnosis improves treatment outcomes by allowing for timely and appropriate management strategies, leading to less invasive and more effective treatments and better prognoses for affected individuals.

Early detection of HPV faces several challenges. The lack of symptoms often leads to delays in detection unless routine screening is performed, causing many infections to go unnoticed until complications arise. Screening methods like Pap smears and HPV DNA tests have limitations in sensitivity and specificity, resulting in false negatives and positives that can cause unnecessary anxiety or delayed treatment. Access to HPV screening tests may be limited in certain regions, particularly low-resource settings or underserved communities, reducing the effectiveness of early detection. Stigma associated with sexually transmitted infections, including HPV, and cultural barriers can deter individuals from seeking screening or disclosing their infection status. Even with early detection, ensuring adherence to follow-up and treatment protocols is challenging, impacting the effectiveness of early intervention. Additionally, coordinating HPV vaccination programs with screening efforts is crucial but complex, as ensuring high vaccine coverage alongside effective screening is essential for maximizing public health impact (Paunović et al. 2023; Zambrano-Ríos et al. 2019).

9.6 Current Diagnostic Strategies for HPV

The underlying purpose of the diagnosis may significantly impact the method utilized to diagnose HPV infection. These objectives may include determining whether HPV is present, whether an infection is current, identifying the HPV type or types linked to the disease, and assessing the level of cellular atypia. HPV could be isolated from productive infections if propagated in vitro using clinical samples. However, high-grade dysplasia and malignancies do not create infectious viruses, making their use restricted.

Population-based research studies may benefit from serologic tests in the enzyme-linked immunosorbent assay (ELISA) format that tracks the antibody response to L1 in VLPs. Anti- E6 and/or E7 serum antibodies are found in the majority of patients with HPV16-positive oropharyngeal carcinoma, cervical malignancies, but not premalignant lesions.

However, consistent, repeatable, and sensitive molecular tests have been created to identify HPV DNA and RNA in cervical swabs and biopsy samples; the FDA has authorized a number of these assays. Methods for these tests include real-time PCR, microarrays, synthetic RNA probes that capture viral DNA, and PCR consensus primers (or other amplification methods) that may be used in combination with a reverse line blot for specific hybridization. While some tests identify E6 or E7 DNA or RNA, others identify L1 DNA sequences. If the objective is to identify as many illnesses as possible, the most sensitive tests could be the better choice.

American cervical cancer screening guidelines recommend cytology-based screening for women aged 21–65 every 3 years or co-testing with cytology and an FDA-approved HPV test every 5 years. Women under 30 should only be screened by cytology due to high HPV- infection frequency. Low-cost screening technologies could make population-wide screening affordable in underdeveloped countries. HPV tests can reduce cervical cancer death rates in women over 30, but their efficacy is limited by cytology sensitivity or acetic acid use.

Despite the inability to culture HPV in the laboratory from clinical specimens, and the inadequacy of immunologic assays for HPV detection, cytology and histology have been the primary diagnostic tools. Recently, molecular methods for detecting HPV DNA sequences in clinical specimens have been introduced. The laboratory diagnosis of HPV includes screening tests, Colposcopy, Biopsy, Imaging tests, HPV genotyping, Visual inspection with Acetic acid (VIA), and Molecular tests, etc. (Hpv Feild Virology 2013).

9.7 Conventional Method

9.7.1 Colposcopy and Acetic Acid Test

Colposcopy is an outpatient procedure conducted by specially trained clinicians using a low- powered microscope known as the colposcope. This procedure involves the examination of the cervix, vagina, and occasionally the vulva following the application of acetic acid solution. It also includes the collection of colposcopies-directed biopsies from all lesions suspected of indicating neoplasia. The assessment of colposcopy findings involves grading based on the degree.of acetowhite.lesion, surface contour, mosaic pattern, and punctuation. More pronounced abnormalities. in these.parameters are indicative of the severity. of the lesions (Sarian et al. 2005).

9.7.2 *Acetic Acid Test*

Acetic.acid application may increase suspicion, particularly in lesions that lack distinctive characteristics. The technique involves placing a 3–5% acetic acid–moistened gauze pad on suspected lesions of the penis, cervix, labia, or perianal area for 5–10 min. This method helps make previously inconspicuous, flat genital lesions visible, which might otherwise be challenging to assess. Genital warts, dysplastic tissues, and neoplastic tissues exhibit a white appearance, known as acetowhite. However, false-positive results are common and can arise from factors causing parakeratosis, such as candidiasis, psoriasis, lichen planus, healing epithelium, and sebaceous glands. Due to these limitations, the acetic acid test is not recommended for routine screening. Nevertheless, it can be utilized to visualize subclinical genital HPV-associated lesions, pinpoint lesions for targeted biopsy, and delineate lesions during.surgical therapy (Sarian et al. 2005; Carr and Sellors 2004; Goel et al. 2005; Hasanzadeh et al. 2011).

Colposcopy screening should only be done when specific populations, such as women living with HIV, transplant recipients with weakened immune systems, and those who have had three consecutive unsatisfactory samples, require special care. This also applies to individuals who have had three tests showing borderline nuclear changes in squamous cells or one test showing borderline nuclear changes in endocervical cells, requiring a colposcopy referral. Further examination is necessary if cervical cytology reveals malignant or suspicious cells even the cervix appears clinically normal. A colposcopy referral is mandated after two tests indicate mild dyskaryosis. An immediate referral for colposcopy is crucial if one test shows moderate or severe dyskaryosis, with at least 90% of these women required to be seen in a colposcopy clinic within 4 weeks. An urgent colposcopy referral is a must if one test indicates possible invasion or glandular neoplasia, with 90% of these cases should be examined within 2 weeks (Menon et al. 2018).

9.7.3 *"Pap Smear: Evolution, Limitations, and Diagnostic Challenges in Cervical Cancer Screening"*

The primary method for detecting high-risk HPV remains the Papanicolaou-stained (Pap) smear, a screening tool introduced by pathologist George Papanicolaou in 1949. The Pap smear specifically targets changes in cells within the transformation zone of the cervix, often associated with HPV infection. This system aimed to provide a uniform and advanced understanding of cervical neoplasia, incorporating statements about specimen adequacy into the report. The 2001 classification categorizes squamous cell abnormalities into four distinct types: AtypicalllSquamousllCells (ASC), Low-GradellSquamous.Intraepithelial Lesions (LSIL), High-Grade. Squamous IntraepithelialkhLesions (HSIL), andkkSquamous.Cell Carcinoma. (Menon et al. 2018; Sahebali et al. 2003).

The ASC category, previously termed Atypical Cells of Undetermined Significance (ASCUS), was refined in the Bethesda System 2001 to include subcategories: Atypical Squamous.Cells of UndeterminedkSignificancekj(ASC-US) and AtypicallSquamouskkCells cannot ExcludellHSILll (ASC-H). The LSIL and HSIL categories were retained, representing low-grade lesions generally associated with transient.HPV.infection and high-grade.lesions consisting of. moderate and severe. dysplasia and.carcinoma in situ (Depuydt et al. 2008; Donders et al. 2013).

Limitations of the Pap smear procedure include inadequate samples (8% of specimens), false- negative rates (up to 20–30%), and potential distortions due to clumping of cells, contamination by other cervical specimen contents, or exposure to air before fixation on the slide. In response, the Clinical Laboratory Improvement Act (CLIA) of 1988 set national guidelines, restricting technicians from reading more than 100 slides per day and mandating manual rescreening of 10% of negative satisfactory smears to reduce false-negative results (Crane 1987; Ehrmeyer 2013; Depuydt et al. 2008).

The Monolayer.cytopathology, a recent advancement in Pap smear specimen collection and processing aims to reduce false-negative results. In these methods, specimens are collected in a preservative solution, enhancing cellular preservation by immediate fixation. The use of a cervical brush, providing nearly twice as many epithelial cells as other devices, ensures a uniform monolayer creation. Slides are prepared under Cytology Laboratory control, preventing uneven manual smearing and eliminating drying artifacts, along with the removal of contaminating substances (Crane 1987; Donders et al. 2013).

Two FDA-approved liquid-based monolayer cytology methods, the PrepStain system and the ThinPrep Pap Smear method, differ in preparation but share underlying principles. The PrepStain system employs an ethanol-based preservative, employing density gradient centrifugation for enrichment and sedimentation on an adhesive-coated slide. The ThinPrep method uses a buffered alcohol preservative, with high-speed rotation ensuring uniform sampling (Crane 1987). A vacuum draws the sample through a filter, and cells are transferred to a glass slide for staining (Shi et al. 2024).

While monolayer cytology increases the cost of a standard Pap smear, over 50 peer-reviewed publications advocate its use for improved detection of precancerous lesions. Studies comparing monolayer cytology to conventional Pap smear cytology reveal statistically significant diagnostic sensitivity improvement across all disease categories, with a 4–11% increase in epithelial cell abnormality detection depending on the patient population. Specimen adequacy also improves, with studies reporting an 11% increase in satisfactory slides and a 29% reduction in limited slides (Conley et al. 2013).

The FDA-approved AutoPap 300QC and PapNet devices aim to ensure consistent and objective Pap smear evaluation without fatigue, using computerized systems to display potentially abnormal cells for analysis. Disputes exist over the accuracy gained through these automated systems, especially considering the additional expense (Shi et al. 2024).

An alternative approach to enhance Pap smear diagnosis is direct staining for HPV using the BenchMark automated Modular System. This system performs immunohistochemistry stains on ThinPrep Pap Smear cytology samples, detecting high-risk HPV genotypes 16, 18, 31, 35, 45, 59, 51, 52, 39, 33, 56, and 70, as well as low-risk genotypes 6, 11, 42, 43, and 44. The INFORM probes indicate HPV infection based on staining intensity, ranging from pale blue to blue-black (Klinkhamer et al. 2003; Conley et al. 2013; Shi et al. 2024; Crane 1987).

9.7.4 Histopathology and In Situ Methods

Patients exhibiting abnormal Pap smear results without a visible cervical lesion are typically subjected to colposcopy and colposcopy-directed biopsy. The procedure involves the application of a 3% acetic acid solution to the cervix, followed by examination under 10- to 15-fold magnification using bright filtered light. This method allows for the visualization of acetowhite areas and specific vascular patterns indicative of dysplasia or carcinoma. Although colposcopy is effective in identifying low-grade and high-grade dysplasia, it is unable to detect microinvasive disease. When no abnormalities arellobserved or the entirellsquamocolumnar junction is not visible, akkcervical cone biopsy is performed (Brown and Garber 1999).

Diagnostic confirmation of most cases can be achieved through biopsies conducted after applying acetic acid. Examination of these tissue samples shows distinctive pathological signs of HPV infection. These include a thickening of the epithelium (acanthosis) and the presence of degenerative cytoplasmic vacuoles (koilocytosis) in fully mature keratinocytes with irregular nuclei. Furthermore, various staining methods can be employed to detect HPV antigens or genetic material using both monoclonal and polyclonal antibodies. These antibodies detect HPV common antigen, expressed broadly among different HPV subtypes. The detection is achieved through peroxidase-anti-peroxidase immunocytochemical staining, typically confined to the nucleus of infected cells (Brown and Garber 1999; Monsonego et al. 2001; Conley et al. 2013) (Weintraub 1997).

In situ hybridization techniques with probes labeled using radioisotopes or chemically reactive ligands are employed to demonstrate HPV, DNA, or RNA in biopsy tissues. This method allows for the localization of HPV nucleic acid sequences within individual cells while preserving cell and tissue morphology. In situ methods can be nonamplified, target amplification by PCR, or signal amplified (Weintraub 1997). HPV detection using nonisotopic probes and enzymatic techniques is easier to interpret than fluorescence. The. GenPoint system is an automated, catalyzed signal amplification system used for immunohistochemical detection of HPV in formalin-fixed, paraffin-embedded biopsy tissue sections. This system can detect as few as 1 to 2 copies of the target sequence per nucleus, exhibiting higher sensitivity compared to routine nonamplified immunoenzymatic procedure (Linder and Zahniser 1997; Weintraub 1997).

9.8 Molecular Techniques

9.8.1 Direct Nucleic Acid Detection Tests

Nucleic acid-based high-risk human papillomavirus (hrHPV) testing is a crucial component of cervical cancer screening. This method focuses on detecting the genetic material of HPV rather than relying on viral load, as the persistence of HPV infection, not the quantity of viral copies, is linked to cancer development. hrHPV testing identifies the presence of HPV DNA or RNA in cervical cells, emphasizing the importance of detecting persistent infections rather than viral load, which is critical in HPV-related carcinogenesis. These tests must detect viral genomes at very low copy numbers, akin to tests for viruses like HIV and hepatitis C, but with a focus on persistent HPV infections (Weynand et al. 2010; Gustavsson et al. 2009). Clinical validation involves comparing test results with cervical cytology to ensure a correlation with clinical outcomes. Extensive clinical validations, such as the ALTS (ASCUS/LSIL Triage Study) and ATHENA (Addressing the Need for Advanced HPV Diagnostics) studies, have established the clinical value of these tests, providing data on the prevalence of cytologic abnormalities, hrHPV positivity, and the incidence of cervical intraepithelial neoplasia (CIN2 or greater). A variety of FDA-approved assays for HPV nucleic acid detection are available, each with unique methodologies, and these tests are analytically and clinically validated, demonstrating proven reproducibility, clinical sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) (Andrei et al. 2022; Weynand et al. 2010; Siqueira et al. 2024).

HPV is undetectable using traditional viral culturing methods due to the lack of cytopathic effects. Direct detection techniques like immunohistochemistry and electron microscopy are inefficient due to background interference and other inefficiencies, and serologic detection of anti-HPV antibodies is limited because HPV immunity is not fully characterized. Molecular tests offer capabilities not possible with other methods, such as analyzing various specimens and identifying specific hrHPV genotypes. FDA-approved tests are optimized for routine cytology specimens, including swabs and liquid cytology media, and are seamlessly integrated into laboratory workflows. Evidence-based consensus guidelines incorporate molecular HPV testing for managing women with cervical abnormalities, and laboratories must use assays that are validated and meet the required standards for clinical practice (Siqueira et al. 2024). The clinical impact and public health implications of hrHPV testing are significant. Early detection of hrHPV can prevent the progression to cervical cancer by identifying and monitoring precancerous lesions, reducing morbidity and mortality associated with HPV-related cancers. Screening programs are cost-effective and reduce the overall healthcare burden. Public health initiatives, including vaccination and screening, rely on accurate and reliable hrHPV testing for effective implementation. Accessible screening ensures equal opportunities for early detection and treatment across diverse populations, and efforts to overcome challenges related to accessibility and awareness are essential for maximizing public health benefits (Siqueira et al. 2024; Weynand et al. 2010; Coutlée et al. 2005) (Table 9.1).

Table 9.1 Commercially available FDA-approved hrHPV nucleic acid detection tests

Test name	Type	FDA approval year	HPV types detected	Specimen types	Indications	Methodology	Limitations
Hybrid Capture 2 High-risk HPV DNA test (hc2)	DNA-based screening	2003	13 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)	hc2 DNA Collection Device, biopsies, broom-type device, PreservCyt solution	(1) Screening patients with ASCUS for colposcopy referral (2) Cotesting with Pap test for women >30 years	Nucleic acid hybridization and chemiluminescent signal amplification detected by luminometer (RLUs)	No internal control, potential cross-reactivity with low-risk HPV types, and other untargeted HPV genotypes
Cervista HPV HR	DNA-based screening	2009	14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)	ThinPrep Pap test PreservCyt solution samples	(1) Screening patients with ASCUS cervical cytology for colposcopy referral (2) Cotesting with Pap test for women >30 years	Uses Invader chemistry for signal amplification and detection of target sequences using oligonucleotide mixtures	Preprocessing treatment with glacial acetic acid can affect assay performance, cross-reactivity with HPV types 67 and 70
Cervista HPV 16/18	DNA-based genotyping	Not specified	hrHPV types 16 and 18	Cervical specimens	(1) Adjunctive use with Cervista HPV HR test and cervical cytology for women >30 years (2) Patients with ASCUS cervical cytology	Uses Invader chemistry for qualitative detection of DNA from HPV types 16 and 18	Similar limitations to Cervista HPV HR, including impact from glacial acetic acid and cross- reactivity

Cobas HPV test	DNA-based screening/ genotyping	2011	14 hrHPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), with separate channels for types 16 and 18	PreservCyt solution collected with an endocervical brush or spatula	(1) Screening patients >21 years with ASCUS cervical cytology for colposcopy referral (2) Assess presence of hrHPV genotypes 16 and 18 (3) Adjunctive screening for women >30 years	Automated specimen preparation, PCR amplification, and real-time detection using cobas 4800 system	None reported by manufacturer
APTIMA HPV assay	E6/E7 mRNA-based screening	2011	E6/E7 mRNA transcripts of 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)	PreservCyt solution, broom-type, cytobrush, or spatula devices	(1) Screening patients >21 years with ASCUS cervical cytology for colposcopy referral (2) Adjunctive screening for women >30 years	Target capture, transcription- mediated amplification, and hybridization protection assay using TIGRIS DTS system	Risk of cross-contamination using TIGRIS DTS, cross- reactivity with low-risk HPV types 26, 67, 70, 82
APTIMA HPV 16–18/45 Genotype assay	E6/E7 mRNA-based genotyping	2012			(1) Testing samples with APTIMA HPV assay- positive results for hrHPV types 16, 18, 45 in patients >21 years with ASCUS cytology (2) Adjunctive use with cervical cytology for women >30 years with APTIMA HPV assay- positive results	Similar methodology to APTIMA HPV Assay, with focus on genotyping for types 16, 18/45	Similar limitations to APTIMA HPV Assay, including cross-reactivity and risk of cross- contamination

9.8.2 *Signal Amplification DNA-Based Assays: The Hybrid Capture System*

Signal-amplification tests, through the amplification of the detection signal without altering the initial amount of nucleic acids in samples, have the capacity to identify lower quantities of DNA compared to direct methods. The signal amplification test called Hybrid Capture II (HC-2), created by Digene Corporation, USA, is the only method approved by the US Food and Drug Administration for identifying HPV in exfoliated cells. Inithis procedure, exfoliated cervical cells are collected using a conical cytobrush from the Digene Cervical Sampler provided in the specimen collection kit, then resuspended in Sample Transport Medium (STM) and stored at room temperature for up to 2 weeks. Exfoliated cells are obtained using a plastic spatula and an endocervical cytobrush, and they are subsequently resuspended in 10 mL to 20 mL of liquid cytology medium (Preservcyt or AutoCyte). An alternative method is also employed. Both collection techniques produce exceptional test results, and cells can be preserved in these medium at room temperature for a maximum of 4 weeks (Coutlée et al. 1997).

The samples were prepared using sodium hydroxide to denature DNA, followed by hybridization of two nonisotopic single-stranded long RNA probes in a solution. Mixture probe A- identifies five low-risk types, namely, 6, 11, 42, 43, and 44, while mixture probe B detects 13 high-risk types which are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HC-2 is being considered as most typically use high-risk probe cocktail. Products obtained from hybridization are then transferred into wells coated with antibody in microplate, these antibodies are used in order to recognize specific DNA/RNA hybrids. The quantification of the product, i.e., bound hybrids which are produced by the fusion of two hybridomasis achieved by adding an alkaline-phosphatase-labeled monoclonal antibody against DNA/RNA-hybrids, addition of a chemiluminescent substrate. The emitted light is measured as relative light units (luminometer), and the quantity of bound hybrids is proportional to the intensity of the emitted light. Results are expressed as the ratio of specimen reactivity to the mean of the positive control, with a specimen considered positive if its reactivity is at least equal to that of the positive control (Söderlund-Strand et al. 2005; Iftner and Villa 2003).

Hybrid Capture-2 system has undergone thorough validation, detecting various high-risk types in a single reaction and utilizing a acceptable format that minimizes potential contamination present in gene amplification assays. It does not demand extensive training, is not subjective, and demonstrates reproducibility with an intra-assay coefficient of variation below 5%, unlike cervical cytology. The homogeneous- hybridization reactions employed in HC-2 are faster than heterogeneous tests, particularly when single-stranded probes are used, making them more suitable for diagnostic laboratories and offering quicker results. However, HC-2 does not account for sample quality based on cell content. It can detect as minimum as 1 pg/mL of target DNA (5000 genome copies per test) (Söderlund-Strand et al. 2005).

Despite the lack of standardization in the PCR assays employed, multiple studies have evaluated Hybrid Capture's specificity and sensitivity in comparison to histology and PCR. The results show a good agreement (kappa values above 0.6) between the two methods. These investigations have shown that the high-risk probe mixture can react with HPV types that aren't in the mix, which could include low-risk types 6, 42, 53, 54, 62, 66, 67, 73, CP6108, and CP8061.

Recent assessments of a variety of clinical investigations have demonstrated that HC-2 has a sensitivity of 90% when it comes to detecting high-grade lesions. Using rapid.capture devices, HPV-DNA testing .could be. automated, thereby combining the processes of sample processing and testing. By utilizing oligonucleotide probes that have been tagged with biotin, the third-generation Hybrid Capture system (HC-3) is expected to successfully capture the DNA sequence of interest while simultaneously .minimizing the background. Noise caused by endogenous DNA/RNA hybrids. Because blocker probes improve the specificity of tests, it is reasonable to expect that HC-3 will be more specific than HC-2100.

9.8.3 Genomic Amplification for HPV Detection and Typing

PCR stands out as a potent tool for conducting epidemiological investigations into HPV infection or cervical cancer. While nucleic acid amplification techniques generally exhibit lower analytical sensitivity than HC.-2, PCR has now become the gold standard for HPV research. The natural history of HPV infection has been elucidated more comprehensively through PCR, overcoming the initial challenges of misclassifying HPV status that confounded the scientific community regarding HPV's role in cervical cancer. Consensus PCR, characterized by high sensitivity, effectively detects most genital HPV types. This method allows for variant analysis of HPV types and facilitates the identification and characterization of .novel HPV types. By incorporating a reverse transcriptase step, PCR enables the analysis of HPV expression in clinical specimens. Notably, a significant correlation has been established between the level of expression of E6 and the grade of CIN (Perkins et al. 2023).

While type-specific PCR tests are impractical for detecting HPV infection in clinical specimens due to the numerous types involved in genital diseases, consensus PCR assays have been developed to amplify the majority of known and novel anogenital HPV genotypes in a single reaction. Subsequent typing involves techniques such as hybridization with type-specific oligonucleotide probes on filters, homogeneous hybridization reactions with RNA probes in the SHARP system (Digene Corporation, USA), restriction fragment length polymorphism, or DNA sequencing (7). Four consensus assays target conserved sequences in the HPV L1 gene, namely, GP5+/GP6+,MY09/MY11/HMB01,PGMY09/PGMY11, and SPF1/SPF2 consensus primer sets, capable of amplifying a broad spectrum of genital HPV types (7,9,13,21). While these assays now use reverse hybridization for the nonisotopic genotyping of HPV amplicons, none are currently commercialized or

standardized for routine clinical use. Future studies will need to compare their performance with Hybrid Capture in clinical trials (Gravitt et al. 1998).

The type of sample tested (fresh tissue or fixed tissue), the size of the amplimer, the5use of degenerate versus nondegenerate primers, the number of primers in the mixture, and the sequence variation at the primer sites can affect consensus PCR assays. The GP5+/GP6+ test can detect 10 to 200 copies of at least 19 genotypes, depending on the gene. Other consensus assays, such as MY09/MY11/HMB01 and PGMY09/PGMY11, amplify a broad spectrum of HPV genotypes with varying levels of sensitivity. The latter set of primers aims to improve sensitivity, specificity, and reproducibility by eliminating degeneracies present in other assays. The SPF system and PGMY primers, when combined with line blot or line probe, can genotype 25 or 37 types, respectively. Recent comparisons between SPF and PGMY systems have shown high overall agreement for detecting HPV-DNA, with a kappa value of 0.86. Additionally, over 96% concordance was observed in genotyping between the two assays, with some types showing differential detection efficiencies. The forthcoming comparison of these new consensus PCR assays with HC-2 will be crucial for establishing the value of PCR and typing in clinical settings (Söderlund-Strand et al. 2005; Zygouras et al. 2023; Perkins et al. 2023).

9.8.4 Detection of Integrated HPV Genome in Human DNA

In low-grade lesions, DNA molecules from high-risk HPV types typically exist in an episomal state. However, in high-grade lesions and invasive tumors, these DNA molecules are predominantly integrated into the human genome. An integration event interferes with the E2 open reading frame, causing E6 and E7 oncoproteins to be produced uncontrollably. HPV-16 integration precedes microinvasion in high-grade dysplasia. Southern blots show HPV incorporation but are insensitive. Real-time PCR tests .amplifying the.E2 and E6 genes measure the episomal to integrated HPV genome ratio. The usefulness of these tests in identifying women at risk for cervical lesions needs additional study (Bønløkke et al. 2024).

9.8.5 ViraliLoad Evaluationiby Real-Time PCR

Quantifying viral HPV-DNA in clinical specimens has the potential to enhance the clinical applicability of HPV detection methods. However, the development of truly quantitative PCR assays for HPV quantification has been limited. By monitoring fluorescence during the amplification step, real-time PCR techniques accurately quantify target DNA copy numbers. Conventional PCR cannot monitor in real time. Real-time PCR uses fast thermocycling and fluorescence detection. Various formats, such as hydrolysis probes (Taqman assays), fluorescence resonance energy transfer-based assays, and assays using molecular beacons, can be employed for

real-time amplification and detection of HPV-DNA. Over a wide target concentration range, these assays yield linear findings. HPV-DNA and a human gene (e.g., beta-globin) can be amplified in each specimen, and the signal can be compared to a titration curve of HPV and cellular DNA. Based on beta-globin amplification's cellular DNA inference, HPV-DNA signal can be altered. Following that, the viral load is expressed as HPV-DNA copies per cell (Téblick et al. 2024).

However, the measurement of viral load has challenges, as sampling high-grade cervical lesions may yield more cellular material, potentially leading to an artificially higher HPV viral load (24). A low-stringency PCR assay, utilizing nonstringent amplification reactions with primers GP5/GP6, consistently generates a specific band from human DNA. This assay allows the calculation of the HPV copy number by determining the ratio of signals between HPV-DNA and human DNA bands (Téblick et al. 2024; Xia et al. 2021; Cricca et al. 2007).

9.8.6 HPV Variant Analysis

Considerable intertypic diversity has been observed among isolates of the same HPV type. HPV variants are characterized by a limited number of point mutations, inducing less than 2% and 5% genetic variation in coding and noncoding regions, respectively, compared to the prototype isolate. Techniques such as single-strand conformation polymorphism or PCR sequencing can be employed to analyze HPV variants. Single-strand conformation polymorphism analysis, suitable for large-scale testing, is less discriminating than sequencing in identifying HPV variants. The classification of variants often involves scrutinizing the Long Control Region (LCR), where the highest variability is typically encountered. There are polymorphisms present in the coding regions of HPV, including the E6 and E7 oncogenes. A higher probability of developing high-grade cervical lesions and anal cancer is associated with the prototype variation of HPV-16, as opposed to the nonprototype variety. In contrast to the European prototype, E6 gene variations have been linked to persistent HPV infection and anogenital disease, and they have also been associated with p53 polymorphism in some cases (Xia et al. 2021).

Biologically speaking, natural variances are distinct. There are several variations of HPV-16 that have nucleotide mutations that change the amino acid that is located at the contact region between the pRB protein and the HPV-16 E7 expression protein. There is a wide range of variation in the cell-mediated inhibition of HPV E6-E7 production among HPV variants 126. DNA-protein interaction is hindered in HPV-18 isolates derived from cervical cancer samples due to the presence of LCR mutations at cellular transcription factor binding sites. By altering the HPV-16 E6 protein, it is possible to alter an HLA-B7 peptide binding epitope. This alteration has the potential to influence the immunological recognition of cytotoxic cells and perhaps give a means of evading immune surveillance (Malagón et al. 2024).

9.9 HPV Serology

Serological testing identifies individuals with current or past HPV infections, but the risk of developing CIN in HPV-1 seropositive women is considerably lower than the risk associated with HPV-DNA detection. The primary serological assay, based on virus-like particles (VLP), involves cloning the L1 gene of HPV into a viral vector to express the capsid, which then self- assembles. The antibody response against viral proteins typically occurs several months after HPV-DNA detection, with antibody levels remaining stable for up to a decade. Concordance between serological results and HPV-DNA detection is generally high, with approximately 60% of HPV-16 DNA-positive women testing seropositive using VLP enzyme- linked immunoassay (EIA). Overall, serology assays achieve a sensitivity level of around 50% for identifying HPV-infected individuals. Serology assays based on antibodies against capsid proteins are mostly type-specific, though notable cross-reactivity exists between types 6 and 11 due to disrupted viral capsids exposing cross-reactive epitopes. Blocking trials with neutralizing monoclonal antibodies like V5 for type 16 improve type specificity. Type-specific immunodominant epitope. Number of lifetime sexual partners, HPV viral load, and HPV persistence affect HPV seroreactivity. Besides viral capsid proteins, E6, E2, E4, and E7 proteins have been used in EIA, Western blot, and radioimmunoprecipitation experiment. When tissue samples are inaccessible, serological testing allows for retrospective investigations on serum banks, which is useful for measuring both present and historical illnesses. Serological tests, however, are neither standardized nor commercialized at this time. Although serology has its uses in vaccination trials and epidemiological research, it has little use in clinical diagnostics (Galati et al. 2024) (Table 9.2).

9.10 Immunological Techniques

The humoral immune response's role in HPV infection remains poorly understood; nevertheless, it is consistently present throughout the malignant process. This ambiguity has prompted the development of various methods and substances for identifying antibodies against both early and late HPV proteins. Among these approaches, recombinant fusion proteins have been employed as antigens in Western blot assays, while synthetic peptides mimicking significant immunogenic B-cell epitopes have been utilized in ELISA (Enzyme Linked Immunoabsorbent Assay). Furthermore, adaptations of these techniques have been implemented to enhance assay specificity and sensitivity. An alternative system for HPV antibody detection involves in vitro protein transcription and translation utilized in radioimmunoprecipitation assays. These varied techniques collectively demonstrate that measuring antibodies against different HPV proteins could serve as valuable biological markers for various types of uterine cervix lesions (Pirttilä et al. 2022).

Table 9.2 Cervical cancer screening tests: Sensitivity, specificity, point of control, advantages, and disadvantages

Test	Sensitivity	Specificity	Lesions association	Advantages	Disadvantages
Colposcopy	63–94%	56–95%	High-grade cervical lesions	Visualizes abnormal areas, directs biopsy	Subjective interpretation, the potential for discomfort
Acetic acid test	Not reported	Not recommended for routine screening	Suspicious lesions	Simple, inexpensive, aids visualization	Low accuracy, not diagnostic
Pap smear	50–90%	80–95%	Precancerous and cancerous cells	Non-invasive, effective, good screening Cost-for	May miss some further testing Abnormalities, requires
Histopathology and in situ	>95%	High	Cancerous and precancerous tissue	Definitive diagnosis	Invasive (biopsy required), time-consuming
Direct nucleic detection (limited use) Acid tests	Variable	Variable	HPV DNA presence	Research tool	Complex, not standardized for clinical use
Signal amplification DNA-based assays (Hybrid capture)	Up to 90% for high-grade lesions	High	High-risk HPV infection	It is more objective than a Pap smear, good for triage	It doesn't distinguish HPV types, doesn't assess cell changes
Genomic amplification for HPV detection and typing (PCR)	High	High	HPV DNA presence and type	Highly sensitive, detects various HPV types	Expensive, complex, not routinely used for diagnosis
Detection of integrated HPV genome	95–97%		HPV integration into human DNA	May predict high-grade lesions (research)	Early stage of research, not yet validated
Viral load evaluation by real-time PCR	Not established for clinical use	Not established for clinical use	HPV DNA copy number	May predict disease progression (research)	Early stage of research, not yet validated

(continued)

Table 9.2 (continued)

Test	Sensitivity	Specificity	Lesions association	Advantages	Disadvantages
HPV variant analysis	Research tool	Research tool	HPV strain identification	May identify high-risk variants (research)	Research tool, not used for diagnosis
HPV serology	~50%	Not applicable	Past or current HPV infection	Identifies past exposure	Low accuracy, not diagnostic for cervical cancer

9.10.1 Detection of Antibodies Against Oncogenic HPV Proteins by Western Blot

The development of recombinant HPV proteins using molecular biology techniques has facilitated the isolation and purification of various viral antigens, enabling the detection of antibodies in diverse bodily samples such as blood, saliva, and cervical mucus. The Western blot assay stands out as the most precise method for antibody detection and has served as a confirmatory test for HIV and HSV infections. One key advantage of the Western blot assay is its ability to identify specific protein bands along with their molecular weights from antigen mixtures. However, due to the denaturing conditions inherent in this technique, only linear epitopes are recognized, and its intricate nature poses challenges in handling large sample volumes (Malagón et al. 2024; Pirttilä et al. 2022; Buckley et al. 1982).

The Western blot procedure involves separating the antigen by molecular weight using polyacrylamide gel electrophoresis, followed by transferring it onto nitrocellulose membranes. Once transferred, the membranes are blocked and cut into strips for incubation with patient samples (serum, saliva, cervical mucus) for 4–16 h. The antigen-antibody complexes are then detected using a secondary antibody complex conjugated with an oxidizing enzyme, such as horseradish peroxidase, resulting in the production of a colored molecule or luminescence. Consequently, the Western blot system has shown a strong association between the presence of HPV-16 E7 antibodies and cervical cancer. However, the presence of anti-E4 antibodies has been a subject of debate, with some studies showing similar distribution among healthy individuals and cancer patients, while others suggest a higher prevalence in cervical cancer subjects. Recent findings indicate that anti-E4 antibodies can serve as markers for HPV infection and early-stage cervical lesions, particularly in CIN I-II lesions. In summary, the Western blot system's ability to detect antibodies against multiple linear epitopes contributes to its high specificity (78%) and sensitivity (67%), making it well-suited for diagnosing various cervical lesions.

9.10.2 HPV Antibody Detection by ELISA

The ELISA serves as a primary clinical diagnostic tool, particularly for screening large populations and conducting molecular epidemiology research. One of its key advantages lies in its capability to recognize both conformational and linear epitopes by antibodies, as it operates effectively under non-denaturing conditions (Buckley et al. 1982; Ghosh et al. 1993).

Widely employed for studying the immunological response to various HPV antigens, ELISA has revealed reports indicating the presence of antibodies against L1 capsid viral proteins in condyloma acuminata and low-grade squamous intraepithelial lesions (LSIL). However, these anti-L1 antibodies are less prevalent in high-grade squamous intraepithelial lesions (HSIL) and nearly absent in cervical cancer

(CC), although controversies exist regarding their frequency in control populations. The development of Virus-Like Particles (VLPs) has enhanced antibody detection sensitivity, particularly for identifying conformational epitopes and neutralizing antibodies. Initially produced from baculovirus and vaccinia virus, VLPs are now generated using a recombinant yeast expression system, contributing to advancements such as the Merck & Co. HPV vaccine. ELISA assays for VLPs exhibit type-specificity and have proven effective in detecting newly acquired HPV infections in previously unexposed women. Moreover, studies have shown that the presence of anti-VLPs antibodies correlates with a higher risk of concurrent severe cervical intraepithelial neoplasia (CIN 3) and cancer, emphasizing their diagnostic significance. In regard to the E2 protein, limited research has been conducted, although studies suggest elevated IgG and IgA responses in CC patients. Conversely, anti-E2 antibodies have been detected in normal subjects and linked to CIN lesions, particularly in women over 40 years old (Ghosh et al. 1993; Buckley et al. 1982; Galati et al. 2024).

E4 protein concentrations have been found elevated in pre-cancerous lesions associated with HPV-16, with antibodies detected in patients with both pre-cancerous lesions and normal individuals, indicating its potential role in active viral replication. Additionally, studies have demonstrated higher proportions of anti-E6 and E7 antibodies in CC patients compared to those with early lesions or healthy subjects, suggesting their utility as markers for cervical cancer, albeit at late diagnostic stages (Ghosh et al. 1993).

Considering the importance of early detection, research into the immune response against viral proteins like E2, E4, and E5, potentially associated with early stages of cervical cancer development, remains crucial. Utilizing diverse systems for recombinant protein production and synthetic peptides can enhance epitope recognition, consequently improving immune assay sensitivity. Furthermore, profiling antibody isotypes against early HPV proteins (E4, E7) has proven useful in identifying infection stages critical to cervical cancer progression. Integrating multiple diagnostic approaches including cytology, DNA testing, and antibody detection offers the prospect of timely and accurate diagnoses, essential for identifying women at high risk of developing cervical cancer. A comprehensive exploration of the humoral immune response against HPV antigens holds promise for developing highly sensitive and specific diagnostic systems tailored to different high-risk HPV types, as well as monitoring the protective immune response in vaccinated populations with L1 antigen-based HPV vaccines (Buckley et al. 1982).

9.11 The Transition from Traditional Diagnostic Methods to Some Innovative Techniques and Clinical Trails

The oldest method for identifying HPV is the Pap smear, which involves identifying abnormalities in a cervical tissue sample. A more modern technique, Liquid-Based Cytology (LBC), employs a brush device to collect a more uniform sample than the

Pap smear, enhancing sensitivity through better sample absorption. However, LBC is more expensive. Another commonly used method is Visual Inspection with Acetic Acid (VIA), which is cost-effective but has limited specificity, particularly in resource-constrained settings. Additional contemporary techniques include Southern blot, dot-blot hybridization, in situ hybridization, and radiolabelled nucleic acid hybridization assays. While these methods offer high-quality results, they demand substantial sample sizes and exhibit low specificity. Advances in molecular biology methods have the potential to enhance the accuracy of HPV detection and typing (Krchnak et al. 1990).

9.11.1 RNA In Situ Hybridization Is Employed to Identify HPV E6/E7 mRNA

In situ hybridization (ISH) is a methodology employed to survey specific nucleic acid segments within heterogeneous cell populations, particularly in tissue samples. This technique relies on preserving nucleic acids adequately within histologic samples, where a reporter molecule, linked to a complementary strand, binds to the nucleic acid. Detection of the reporter molecule enables the localization of RNA sequences, offering insights into gene expression levels. In the context of cervical cancer development, the overexpression of HPV E6/E7 oncoproteins, measurable as E6/E7 messenger RNA (mRNA) transcripts, is crucial. RNA in situ hybridization (RNA-ISH) is a highly sensitive and specific method for detecting HPV E6/E7 mRNA by pinpointing individual cell signals in clinical samples.

A study conducted by Pandey, Bhosale, and Mahimkar in 2018 utilized the RNAscope® assay kit, a technology based on RNA in situ hybridization for simultaneous detection of E6/E7 mRNA of seven high-risk HPV subtypes (HPV 16, 18, 31, 33, 35, 52, and 58). The tissues were initially fixed in formalin and embedded in paraffin. Each tissue section was then divided into three parts: the first for HPV testing, the second for a positive control for Ubiquitin C (UBC), and the third for a negative control for the bacterial gene dapB. The reporter molecule employed was diaminobenzidine present in Brown Reagent Kits (RNAscope), resulting in a prominent spot when target gene expression was detected (Buckley et al. 1982).

To assess treated samples, the researchers employed the UBC test to evaluate the presence of hybridizable RNA. If the RNA quality was insufficient, the UBC slide yielded a negative result, leading to the disqualification of the sample. Evaluation of the dapB test was performed in cases of nonspecific staining. Finally, negative cases with weak staining were subjected to HPV scoring, and a positive HPV test result was determined by punctate staining within the cytoplasm or nucleus of malignant cells (Stacey et al. 1992).

9.11.2 Genosensors Designed for the Early Identification of High-Risk HPV DNA Associated with the Development of Cervical Cancer

Genosensors play a crucial role in the early identification of high-risk HPV DNA, specifically HPV types 16 and 18, which are linked to a heightened risk of cervical cancer. The initial stage of the viral attack involves the creation of E6 and E7 proteins, disrupting normal cell function and fostering the development of malignant keratinocytes. These infected cells, though exhibiting uncontrolled growth to avoid cell death, may have minimal viral concentration. Genosensors, especially DNA-based ones, emerge as a viable solution for early HPV detection, particularly in precancerous stages.

These genosensors can be categorized based on their diagnostic nature, including electrochemical, optical, fluorescent, piezoelectric, magnetic, aptameric, and immunosensors. Electrochemical biosensors, pioneered by Vernon et al. in 2003, utilize a gold electrode with an assembled monolayer of oligonucleotides specific to HPV genotypes, allowing for the identification of 21 types of HPV. Continuous improvement involves modifying the electrode composition by introducing nanomaterials to enhance specificity and identify more prevalent or dangerous HPV strains.

Fluorescent sensors provide in situ evidence of HPV strain existence, utilizing organic nanocrystals as labels for quantifying HPV genotypes. The manipulation of nanocomponents optimizes infection detection. Magnetic biosensors, a multilayered system, rely on specific biological probes immobilization to interact with samples containing HPV traces. Piezoelectric biosensors, stemming from electrochemical and optical transducers, detect nucleic acids through a sequence-specific technique. Constructed around a piezoelectric crystal substrate, these biosensors accumulate electrical charge due to mechanical stress. Additionally, Surface Acoustic Waves (SAW) utilize Bis-peptide nucleic acid (BisPNA) probes for detecting HPV DNA through double-stranded target DNA capture (Lee et al. 2009; Mahmoodi et al. 2019).

9.11.3 Advanced Electrochemical Biosensor Designed for HPV Detection Based on DNA

An electrochemical DNA biosensor integrates biological and detection components, where DNA hybridization with biosensor materials induces detectable electrochemical alterations, such as changes in light emission or absorption, mass transport, and proton concentration, yielding quantifiable signals. These biosensors offer advantages like rapid detection, affordability, portability, high sensitivity, selectivity, scalability, and user-friendliness compared to other nucleic acid assays. Jampasa et al. (2018) introduced a novel electrochemical DNA sensor utilizing sandwich hybridization to simultaneously identify HPV-16 and 18. The sensor comprises two signaling probes: an unlabeled acpcPNA capture probe (P1) with a peptide backbone,

capable of forming high-affinity, specific antiparallel hybrids with complementary target DNA; and an anthraquinone (AQ) modified acpcPNA probe (AQ-P2). P1 is immobilized onto a Cys/gold-modified electrode via a cross-linking agent, while AQ-P2 is designed to partially complement upstream (ASU) or downstream (ASD) positions on the target DNA sequence, facilitating electron transfer between the electrode surface and the labeled DNA molecule and inducing electrochemical alterations in the biosensor. To validate.

its efficacy, cervical cancer cell lines containing HPV-16 and 18 were utilized, sourced from the Human Genetics Research Group (Mahmoodi et al. 2019; Stacey et al. 1992).

9.11.4 A Novel PCR-Based Urine Test for Detecting and Genotyping HPV-DNA

This approach eliminates the need for cervical sample collection, utilizing only a urine sample. The urine assay, a non-invasive self-collection method, proves to be more convenient and acceptable than traditional cervical sample biopsies. Recent studies have incorporated MY09/11 primers for HPV DNA detection. (Tanzi et al. 2013) introduced a novel set of primers positioned similarly to the MY09/11 consensus primer pair, leading to increased sensitivity and detection of a higher number of HPV genotypes through PCR.

To assess the efficacy of this updated PCR assay, cervical and urine samples from HPV- infected women (high-risk group) were tested. Urine samples from 107 infected women were collected and subjected to DNA extraction using commercial methods. Sample purity and concentration were assessed with a spectrophotometer. HPV detection in DNA samples involved PCR amplification using degenerate primer pair ELSI-f and ELSI-r, with each run including positive and negative controls. Genotype analysis was performed through restriction fragment length polymorphism on the PCR products, and the molecular weight of the generated fragments facilitated the identification of new genotypes. In urine samples, 31 different HPV types were identified, compared to 29 HPV types in cervical samples. Notably, HPV-51 and 73 were exclusive to cervical samples, while HPV-39, 59, 69, and 85 were identified solely in urine samples (Tanzi et al. 2013).

9.11.5 The Molecular Technique for Detecting HPV in Semen Samples

All previous HPV detection tests primarily targeted women. In 2018, the Centers for Disease Control and Prevention emphasized the significant role men play in HPV transmission, highlighting the lack of an approved test for HPV in men. This

Table 9.3 Some innovative techniques

Molecular application for Hpv detection	Number of Hpv genotype detected	Hpv genotype detected exclusively compared with cervical sample	Qualitative feature
RNA in situ hybridization is employed to identify HPV E6/E7 mRNA	Seven high-risk HPV subtypes (HPV 16, 18, 31, 33, 35, 52, and 58)	E6/E7 mRNA	High specificity Good sensitive
Advanced electrochemical biosensor designed for HPV detection based on DNA	Two (HPV-16 and 1 8)	None	Rapid detection Low cost Portable High affinity High specificity Good sensitivity Good selectivity Compatible with mass production
A novel PCR-based urine test for detecting and genotyping HPV-DNA	31 different HPV types	HPV-39, 59, 69, and 85	Non-invasive Self-sample collection
The molecular technique for detecting HPV in semen samples	16, 18, 44, 31, 73 HPV genotypes	None	Non-invasive Self-sample collection

molecular approach offers a convenient method for collecting and detecting HPV exclusively in males. The DNA was extracted from total semen, including an aliquot from the swim-up procedure (SU fraction), using the QIAamp Mini Kit (Qiagen). The detection and genotyping process involved a 10- microliter DNA sample for PCR-reverse transcriptase hybridization, utilizing the INNOLiPA HPV Genotyping Extra kit (Fujirebio Europe) as per the manufacturer's instructions. This kit facilitates specific probes for HPV genotypes 16, 18, 44, 31, and 73. The total semen and SU fraction underwent lysis to extract all semen components through the Cell Culture DNA kit (Qiagen), following the manufacturer's instructions with some adjustments. The protocol was meticulously tuned, monitoring each step for effective cell and sperm lysis (Capra et al. 2019) (Table 9.3).

9.12 Isothermal Amplification Techniques as Rapid Alternatives to PCR

PCR is a versatile nucleic acid amplification technique widely used in biomedical research and HPV diagnostics due to its high sensitivity, specificity, and ability to multiplex. However, it requires a thermal cycler, is time-consuming, and is sensitive to inhibitors. To address these drawbacks, isothermal amplification techniques (IATs) were developed. IATs operate at a constant temperature, using special

polymerases with strand displacement abilities, achieving similar sensitivities to PCR but in shorter times (20–30 min) and with resistance to PCR inhibitors. This makes IATs suitable for integration with advanced technologies like lateral flow assays and microfluidic platforms (Zhang et al. 2022).

One prominent IAT is loop-mediated isothermal amplification (LAMP), which rapidly amplifies DNA or RNA using Bst polymerase and several primers at temperatures between 55 °C and 70 °C. LAMP has been widely used for detecting bacterial and viral nucleic acids, including HPV. Despite its advantages, LAMP is sensitive to contamination and requires careful primer design. LAMP products can be detected colorimetrically, turbidimetrically, or through more sensitive methods like Real-Time LAMP or digital LAMP. Another IAT, recombinase polymerase amplification (RPA), uses a cocktail of three enzymes to amplify DNA at mild temperatures (37–42 °C) and is particularly useful in simple devices without thermoblocks. However, RPA reagents are only available from a single company, increasing costs and posing supply risk (Zhang et al. 2022).

9.13 Lab-on-a-Chip Technology and Microfluidics: Including Separate Steps into a Single Device

Microfluidic technologies, or "lab-on-a-chip," are revolutionizing biomedical and chemical research by providing low-cost, high-throughput devices that process microliters of solutions. These devices minimize the need for samples and reagents while performing reactions in sealed systems to prevent cross-contamination, making them particularly impactful in DNA diagnostics, including HPV analysis. Innovations in this field include automated high-risk HPV diagnostics that combine machine learning with single-cell droplet PCR and multiplexed microfluidic chips, allowing neural networks to analyze droplet images for HPV presence. Despite the high equipment cost, this approach shows promise for future point-of-care automation. Another advancement is a femtoliter-sized microfluidic hybridization assay that detects HPV at ultralow levels without DNA amplification, using femtoliter-sized droplets and magnetic beads for better sensitivity than commercial kits, though the study lacked negative controls. Paper-based microfluidics, introduced by the Whitesides group in 2007, uses capillary action to transport fluids without pumps or electricity, offering a low-cost, disposable platform for HPV analysis that integrates DNA extraction, LAMP amplification, and lateral flow detection. While this approach showed some false positives, it holds the potential for quick diagnostics in low-resource settings. However, challenges remain, such as the complex and non-standardized manufacturing process, the need for trained personnel and frequent quality checks for integration with other platforms, and issues like high sample retention and higher detection limits in paper-based microfluidics. Examples of innovative applications include the MiCaR platform by Xu et al., which combines microfluidics with RPA and CRISPR-Cas12a for multiplexed HPV genotype

detection within 40 min, and Zhao et al.'s dual-droplet device for detecting HPV16 and HPV18 with ultra-low detection limits in 30 min. These technologies have significant potential to transform diagnostics, especially in low-resource settings, but addressing technical and manufacturing challenges is crucial for broader applications (Surappa et al. 2023).

9.14 Enhancing Selectivity Using CRISPR-CAS System

CRISPR-Cas technology, introduced in 2012 by Jennifer Doudna and Emmanuelle Charpentier (who won the Nobel Prize in Chemistry in 2020), has revolutionized genome editing and molecular biology. Shortly after its introduction, it was applied to molecular diagnostics and biosensing. The CRISPR-Cas system targets and cleaves specific nucleic acid sequences using guide RNA and a catalytically active Cas protein, greatly enhancing the specificity of bioassays. Various Cas proteins, such as Cas9, Cas12, Cas13, and Cas14, can cleave different target molecules, including single-stranded and double-stranded DNA and RNA. This system has been used to detect biomarkers, bacteria, and viruses, including HPV, and even to treat HPV-associated cervical cancer by inducing autophagy and immune activation through CRISPR-Cas (Bartosik et al. 2024).

CRISPR-Cas can identify HPV genotypes at very low copy numbers, often in combination with RPA and fluorescence detection. For instance, Xu et al. developed the MiCaR platform, combining a microfluidic device with RPA and CRISPR-Cas12a for multiplexed detection of nine high-risk HPV genotypes within 40 min, achieving high sensitivity and specificity. Similarly, Zhao et al. created a microfluidic dual-droplet device for detecting HPV16 and HPV18 with ultra-low detection limits, although targeting the L1 gene could lead to false negatives due to HPV integration into the host genome.

Some studies have explored amplification-free CRISPR-Cas assays. Xue et al. introduced a polydisperse droplet digital CRISPR-Cas-based assay for dual detection of HPV18 DNA and SARS-CoV-2 RNA, achieving 100% sensitivity and specificity, though the sample size was small. Despite its promise, CRISPR-Cas technology faces challenges like off-target activity and the need for pre-amplification steps, which extend protocol times. However, combining quick amplification techniques with CRISPR-Cas, as demonstrated by Ganbaatar, who coupled a 20-min RPA reaction with a 10-min CRISPR-Cas12a cleavage, shows potential for use in low-resource setting.

In conclusion, CRISPR-Cas technology offers high specificity and single-nucleotide resolution, but designing precise guide RNAs is crucial due to the sequence variability within HPV. Using techniques that recognize longer sequences or applying CRISPR-Cas where single mismatches are critical can enhance its effectiveness (Surappa et al. 2023; Convery and Gadegaard 2019).

9.15 Lateral Flow Assays and Reverse Dot Blots

Among the many available diagnostic techniques, reverse dot/line blot and lateral flow assays (LFA) have become popular for their simplicity, cost-effectiveness, and speed. Reverse dot blot is valuable for identifying mutations linked to hereditary diseases and cancer-associated genes, while LFAs are primarily used for detecting proteins, especially antigens. The reverse dot blot involves binding gene-specific oligonucleotide probes onto predefined dots or lines on a nylon membrane, followed by hybridization with complementary target DNA, typically labeled PCR products. This method is simple, rapid, accurate, and cost-effective, allowing for the screening of multiple mutations in a single reaction. However, it can suffer from weak signals or false positives due to errors during the conjugation step and visual interpretation of results.

Reverse dot blot has been used for HPV screening since 1998, when a reverse line blot strip assay was developed to detect 27 HPV genotypes. Despite high concordance with previous dot-blot assays, the lack of validation with the gold standard and the elimination of false signals were drawbacks. Lateral flow assays (LFAs), also known as lateral flow tests or devices, are rapid, simple, and low-cost paper-based platforms for detecting target analytes in various samples without specialized equipment. LFAs are commonly used for detecting protein antigens or antibodies and have been adapted for nucleic acid detection. LFAs typically consist of a sample pad, a conjugate release pad, a detection zone with test and control lines, and an absorbent pad. Major challenges include low multiplexing abilities, lower sensitivity, and the need for high-quality antibodies to ensure accurate results.

For instance, Xu et al. developed a dual-color fluorescence-based LFA to detect four common HPV types (6, 11, 16, and 18) by incorporating fluorophore-labeled detection probes in the PCR reaction and immobilizing capture probes at different strip locations. This method achieved high concordance with the GenoArray kit. Similarly, combining LFAs with RPA has shown promise, such as Ma et al.'s study that detected 25 HPV types with high concordance with routine cervical screening.

Recent advancements include the NATflow platform for RPA-based HPV detection, which eliminated the need for DNA extraction and reduced assay time to 45 min, demonstrating high sensitivity and specificity. Another study developed an RPA-based LF strip for detecting HPV16/18 DNA in circulating cell-free DNA from serum samples, showing high sensitivity and specificity but raising questions about the use of circulating HPV DNA as a diagnostic biomarker due to its low abundance in precancerous lesions. Overall, reverse dot/line blot and LFAs offer promising diagnostic capabilities, especially for point-of-care applications, but they also face challenges that require ongoing optimization and validation (Bartosik et al. 2024.

9.16 Nanomaterials Enhancing Assay

Significant advances in nanobiotechnology and nanomaterial science have fueled the development of nanoscale bioassays and biosensors. Nanomaterials, with their unique mechanical, optical, electrical, and magnetic properties due to small dimensions, offer ultralow detection limits thanks to their high surface-to-volume ratios. Various nanostructures, such as gold or silver nanoparticles, carbon nanotubes, graphene oxide, quantum dots, polymeric nanocomposites, and different nanorods or nanowires, are employed as carriers for bioreceptor immobilization or as signal-generating reporters. Often, multiple nanomaterials are combined to create complex architectures within a single assay, aiming for the lowest possible detection limits, though these advancements frequently lack application in real clinical settings. The pursuit of ultrahigh sensitivity is questionable if not validated with clinical material. Additionally, nanomaterials are costly, and their potential negative health and environmental impacts are under scrutiny, with studies reporting their penetration and accumulation in cytoplasm or even cell nuclei.

In HPV analysis, nanomaterials are commonly used, but many studies do not demonstrate the feasibility of clinical material. Instead, synthetic sequences are often spiked into serum to evaluate matrix effects. When cervical samples are used, the number of patients is typically low. For example, Hong et al. employed carboxylic group-functionalized magnetic nanoparticles for an electro chemiluminescent assay detecting HPV16 with a detection limit of 6.8 aM, applied to 27 cervical smears, showing substantial agreement with the hc2 assay. Sun et al. developed a photoelectrochemical biosensor array (PEBA) for multiplexed detection of nine HPV genotypes with a detection limit of 0.1 copies/μl, analyzing 40 clinical samples. A larger cohort of 209 cervical samples validated a nanoparticle-assisted PCR assay (nanoPCR) for detecting HPV16 and HPV18 E6 genes, showing a ten-fold increase in sensitivity compared to conventional PCR and faster assay times (Habibey et al. 2022).

Despite these advances, the benefits of nanotechnology for HPV diagnostics in low-resource settings, in terms of simplicity and cost-effectiveness, remain to be proven. Health and environmental risks also need addressing. Nanomaterials often exhibit lower recognition efficiencies in complex biological environments, slow binding kinetics due to heterogeneous interfaces, and questionable stability of nanomaterial-based surfaces, all of which must be considered when designing biosensing technologies (Habibey et al. 2022) (Table 9.4).

9.17 Conclusion

Over time, the molecular diagnosis of Human Papillomavirus (HPV) has seen significant advancements, transitioning from conventional methods like colposcopy, Pap smear, and histopathology to more sophisticated molecular techniques. The

Table 9.4 Clinical trials regarding novel techniques and biomarkers for HPV diagnosis cancer

Title of study	Study ID, type, and status	Purpose and objectives
A Study to Validate HPV DNA Genotyping and Prognostic Genomic Biomarkers for Diagnosis	**NCT04564989 Sponsor Protocol Number: LCCC 2044** Observational study **Status** Closed for enrolment	Assessment of novel tumour genomic biomarkers of recurrence risk (TRAF3 and CYLD gene defects and HPV integration) in patients with Human papillomavirus + oropharyngeal squamous cell carcinoma (HPV + OPSCC) undergoing curative-intent therapy. (UCSF Human Papillomavirus Clinical Trials for 2025 — San Francisco Bay Area n.d.)
Testing of Real-Time Diagnostic Platform (HPView) for the Detection of High-risk HPV Strains in Urine	**Sponsor Protocol Number: 18-005528** Observational study **Status** Closed for enrolment	Testing of a real-time HPV detection platform (HPView) developed by the researchers to find out whether it performs at a comparable level to the clinical gold-standard. (UCSF Human Papillomavirus Clinical Trials for 2025 — San Francisco Bay Area n.d.)
Patient Self-sampling of HPV to Screen for Cervical Cancer	**NCT05600283 Sponsor Protocol Number: 22-008247** Interventional study **Phase** Not applicable **Status** Closed-enrolling by invitation	1. Validation of a patient self-sampling vaginal collection kit and laboratory testing for the detection of HPV infection 2. Survey of study enrollees to assess the patient perspective on acceptability and feasibility of a self-sampling approach for cervical cancer screening 3. Assessment of the stability testing component to investigate concordance of multiple self-collected Evalyn brush HPV results within the same study subject. (HPV infection - Diagnosis and Treatment - Mayo Clinic n.d.)
Studying Biomarkers in Diagnosing Cervical Lesions in Patients with Abnormal Cervical Cells	**NCT00892866 Sponsor Protocol Number: GOG-0237** Observational study **Status** Closed for enrolment	Study of biomarkers in order to improve the diagnostic ability of cervical lesions in patients with abnormal cervical cells and also improve their prognosis. (UCSF Human Papillomavirus Clinical Trials for 2025 — San Francisco Bay Area n.d.)
Human Papilloma Virus (HPV) Circulating Tumour DNA (ctDNA) in Cervical Cancer	**NCT04574635 Sponsor Protocol Number: ROR1905** Observational study **Status** Open for enrolment	Plasma circulating tumour DNA (ctDNA) is obtained by serial analysis before, during, and following surgery, radiotherapy, chemotherapy, and/or immunotherapy for cervical cancer. This study investigates whether plasma ctDNA is useful for risk stratification, individualized treatment decision making, monitoring of treatment response, and early detection of residual or recurrent disease in patients presenting with cervical cancer caused due to HPV. (HPV Infection - Diagnosis, and Treatment - Mayo Clinic n.d.)

(continued)

Table 9.4 (continued)

Title of study	Study ID, type, and status	Purpose and objectives
OPX Saliva Biomarker Study	**NCT05093400** Observational study **Status** Closed for enrolment	1. In patients with HPV associated oropharyngeal squamous cell carcinoma (HPV (+) OPSCC) but not in HPV (−) OPSCC, oral cavity cancer or normal controls, salivary tumor DNA (stDNA) and the HPV self-test for oncogenic HPV will be detectable 2. The microbiome, virome, and metabolomics will differ among these groups 3. The patient reported risk factors will differ between patients with malignancy and normal controls 4. Dynamic changes in the microbiome and metabolomics will be observed which correspond with patient reported outcomes in patients with oropharyngeal cancer or oral cavity cancer
Investigating Human Papillomavirus (HPV) Infection and HPV-Associated Disease in Indian Men Who Have Sex With Men Who Are HIV-Positive	**NCT04142398** Observational study **Status** Not yet accepting patients **Start date** April 2025 **Completion date** December 2029 (estimated) **Sponsor** AIDS Malignancy Consortium **Participants** Expecting 246 study participants **Last updated** February 2024	**Primary**: Determination of the prevalence of HPV-associated premalignant lesions in the anus and penis in Indian HIV-seropositive men who have sex with men (MSM) **Secondary:** 1. Defining risk factors for prevalent premalignant lesions in the anus and penis in Indian HIV-seropositive MSM 2. Defining the prevalence and incidence of penile and anal HPV infection in Indian HIV-seropositive MSM A questionnaire will be given to the participants and they will undergo the following examinations—a targeted physical and anal clinical exam at months 0, 6, and 12; a penile skin cell and anal swab at months 0, 6, and 12 for cytology, HPV DNA, and CD4+ T-cell count at months 0 and 6 and HIV viral load testing at months 0 and 12 as well as a high-resolution anoscopy (HRA) and penile clinical exam at month 12. (Search for: HPV Infection | Card Results | ClinicalTrials.Gov n.d.)

(continued)

Table 9.4 (continued)

Title of study	Study ID, type, and status	Purpose and objectives
Screening and Identification of Novel Diagnostic and Prognosis Biomarkers on Cervical Cancers	**NCT00854269** Interventional study **Phase** Not applicable **Study start** 2007–01 **Primary Completion (Estimated)** 2008–05 **Study Completion (Estimated)** 2011–01 **Enrolment (Estimated)** 250	1. Arrangement of the platforms to survey the humoral immunity to HPV infection in normal population and patients with CIN lesion or cervical cancer 2. Describing the association between humoral immunity and status and clinico-pathologic items of HPV-infected populations 3. Survey whether humoral immunity is linked with the prognosis of patients with cervical lesions 4. Describing the association between HLA haplotype and humoral immunity in HPV-infected populations The sample taken is a cervical biopsy sample or specimen of hysterectomy. The outcome measure is overall survival and the time frame of study is from disease diagnosis to death (Search for: HPV Infection | Card Results | ClinicalTrials.Gov n.d.)
A Multicenter Cohort Study of HPV Viral Load in Predicting the Prognosis of Women With LSIL in Cervix	**NCT05051852** **Study type** Observational [Patient registry] **Study start (actual)** 2021-09-01 **Primary Completion (Estimated)** 2024-12-31 **Study Completion (Estimated)** 2024-12-31 **Enrolment (estimated)** 1000 **Biospecimen retention:** Samples with DNA **Biospecimen description:** DNA samples from cervix and vaginal fornix & cell samples from cervix	**Primary outcome measure description** 1. Cervical histopathology to collect cervical exfoliated cells performed at baseline for all participants and at 6, 12, and 24-months follow-up for cervical HPV infection or cytology abnormalities women. Colposcopy performed during this process if abnormal cervical lesions are found 2. Human Papillomavirus (HPV) viral load test performed at baseline and at 6, 12, and 24-months follow-up for all participants 3. Cervical Thinprep Cytology Tests (TCT) performed for all participants at the time of baseline and at 6, 12, and 24-months follow-up 4. Vaginal fornix swabs taken from all participants in order to perform vaginal secretion sequencing at baseline and at 6, 12, and 24-months follow-up bioinformatics analysis also performed. 5. Vaginal microbiota tests performed for all participants at baseline and at 6, 12, and 24-months follow-up **Secondary outcome measure description** HPV genotyping tests performed for all participants at baseline and at 6, 12, and 24-months follow-up (UCSF Human Papillomavirus Clinical Trials for 2025 — San Francisco Bay Area n.d.)

(continued)

Table 9.4 (continued)

Title of study	Study ID, type, and status	Purpose and objectives
Effect of HPV Integration on Prognosis of Young Women With CIN2 in China: A Multi-center Cohort Study in China	**NCT05282095** **Study type** Observational **Study start (actual)** 2022-06-01 **Primary completion (Estimated)** 2025-05-31 **Study completion (estimated)** 2025-05-31 **Enrolment (estimated)** 300 **Biospecimen retention:** Samples with DNA **Biospecimen description:** Samples from cervix and vagina containing related cells, tissues and secretions	1. To define the correlation between HPV integration and natural outcome in young CIN2 women 2. To define the prognostic value of different HPV integration status in young women with CIN2 3. To define the relationship between the integration status of different HPV genes in young CIN2 women and the results of vaginal flora and cervical exfoliative cytology **Primary outcome measure description** Following investigations were performed: 1. Cervical histopathology at baseline, 6 and 12-months follow-up for all participants 2. HPV viral integration test at baseline, 6 and 12-months follow-up for all participants 3. Cervical Thinprep cytology tests (TCT) for all participants at the time of baseline, 3, 6, 9, and 12 months follow-up 4. 16SrRNA sequencing of the vaginal secretions at baseline, 3, 6, 9, and 12 months follow-up 5. HPV genotyping tests were performed for all participants at baseline, 3, 6, and 9 months follow-up (Search for: HPV Infection | Card Results | ClinicalTrials.Gov n.d.)

unique structural and genetic complexities of HPV, including its diverse genome and the oncogenic potential of its E6 and E7 proteins, underscore the importance of precise and early detection for effective disease management. Recent approaches, such as RNA in situ hybridization, genosensors, and advanced biosensors, have substantially improved the sensitivity and specificity of HPV detection, particularly in identifying high-risk HPV types associated with cervical cancer. Innovative technologies like PCR-based urine tests, lab-on-a-chip devices, and CRISPR-Cas systems have further revolutionized the field, offering more rapid, accurate, and non-invasive point-of-care diagnostic options. These advancements not only enhance our ability to detect HPV more effectively but also pave the way for personalized and early diagnostic strategies, crucial for early intervention and better clinical outcomes in HPV-related diseases. As research continues to evolve, these emerging technologies promise to play a pivotal role in the future of HPV diagnostics, ultimately contributing to improved public health and cancer prevention efforts.

The future of HPV molecular diagnostics will be driven by new technologies and interdisciplinary methods. CRISPR-Cas systems are poised to offer precise and cost-effective HPV detection, particularly in point-of-care settings. Artificial intelligence (AI) and machine learning (ML) are expected to enhance the analysis of complex data, improving HPV detection, genotyping, and risk assessment. These technologies may also help identify new biomarkers for HPV-related cancers, aiding early diagnosis and personalized treatment. Liquid biopsy provides a non-invasive way to monitor HPV by detecting viral DNA in body fluids, offering real-time insights. Combining molecular diagnostics with HPV vaccination efforts is anticipated to reduce the global burden of HPV-related cancers and refine vaccination strategies. Overall, the integration of advanced molecular techniques, AI, and personalized medicine promises to improve patient outcomes and highlights the need for continued research and innovation.

9.18 Prevention

Primary strategies for preventing HPV infection involve both risk reduction and the development of HPV vaccines. Measures such as using latex condoms and spermicides may reduce the risk of contracting HPV, but their effectiveness is not absolute, as HPV transmission can occur through contact with unprotected body parts like the labia, scrotum, or anus. Despite these preventive efforts, vaccines targeting HPV are currently in phases I and II of clinical trials and are not yet available commercially.

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Chapter 10
Molecular Diagnostics for Mosquito-Borne Viral Diseases: Challenges and Emerging Concepts

Rajendra Kumar Baharia, Soumya Pradhan, Vikas Kumar, and Bhavya Modi

Abstract Mosquito-borne viral diseases, including dengue, Zika, chikungunya, and West Nile virus, pose significant public health challenges worldwide. Molecular diagnostics play a critical role in the early detection and management of these infections. This chapter explores various molecular approaches, including conventional polymerase chain reaction (PCR), real-time PCR, reverse transcription PCR (RT-PCR), loop-mediated isothermal amplification (LAMP), and next-generation sequencing (NGS). A comprehensive review of commonly used PCR primers for detecting specific viral genomes is provided, highlighting their sensitivity and specificity. Despite advancements in molecular diagnostics, several challenges remain, particularly in epidemiological surveillance and field applications. Issues such as sample degradation, cross-contamination, logistical constraints, and limited access to high-throughput technologies hinder effective disease monitoring in endemic regions. Additionally, viral genetic variability and co-infections complicate accurate detection. Emerging concepts, including portable PCR platforms, CRISPR-based diagnostics, and metagenomic approaches, offer promising solutions for rapid and reliable field detection. The integration of molecular diagnostics with surveillance programs is essential for real-time outbreak response and public health interventions. This chapter underscores the need for continuous refinement of molecular tools to improve diagnostic accuracy and field applicability in combating mosquito-borne viral diseases.

Keywords Mosquito-borne diseases · Viral diseases · Zika · Yellow fever · PCR

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10.1 Introduction

Mosquito-borne diseases are significant public health issues that contribute to high morbidity and mortality worldwide. As per WHO, nearly 700,000 people lose their lives each year by Mosquito-borne diseases. The diseases are transmitted by mosquitoes and include malaria, dengue, chikungunya, Zika, yellow fever, and Japanese encephalitis. Due to the favorable climatic conditions, vector-borne diseases occur primarily in tropical and subtropical regions of the world. They are transmitted by blood-feeding arthropods, including mosquitoes, ticks, sandflies, and triatomine bugs. Among the mosquitoes, Aedes and Culex species are the most important vectors responsible for viral transmission. Medically important viruses transmitted by Aedes mosquito include Dengue virus (DENV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Yellow Fever virus (YFV) etc., while Culex mosquito transmits Japanese encephalitis virus (JEV) and West Nile virus (WNV). Ticks also transmit viruses like Crimean-Congo hemorrhagic fever virus (CCHFV), Kyasanur forest disease virus (KFDV), and Tick-borne encephalitis virus (TBEV) (Schmaljohn and McClain 1996). Arboviruses or arthropod-borne viruses are transmitted to humans or animals by blood-feeding arthropods, mainly mosquitoes and ticks. These arboviruses possess two types of transmission cycles: sylvatic cycle in non-human primates and urban cycle in humans. Birds and non-human primates are mainly the reservoir hosts for these viruses. Due to the spillover from sylvatic cycle, they initiate the urban cycles (Weaver 2013). Malaria is caused by Plasmodium parasites and transmitted by Anopheles mosquitoes. Although, it's not a viral-disease, we are discussing it here as per the context of mosquito. It remains one of the most severe public health challenges globally. In 2020, approximately 241 million malaria cases were recorded globally, with 627,000 resultant deaths. Geographic Distribution: The majority of malaria cases (95%) and deaths (96%) occur in the WHO African Region. Pregnant women and children under five are particularly at risk. In 2020, children under five represented 77% of all malaria deaths globally. Malaria remains a major public health problem in India, with significant regional variations in incidence and severity. In 2019, India reported approximately 5.6 million malaria cases and 10,000 deaths, according to the National Vector Borne Disease Control Programme (NVBDCP). High transmission areas include the northeastern states, Odisha, Chhattisgarh, Jharkhand, Madhya Pradesh, and tribal areas. Vulnerable Populations: Tribal communities and rural populations are at higher risk due to limited access to healthcare and preventive measures (World Health Organization (WHO) 2021)[1,2]. *Dengue fever*, caused by the dengue virus and transmitted by Aedes mosquitoes, is endemic in more than 100 countries. Incidence: WHO estimates around 390 million dengue infections occur each year, with about 96 million

[1] National Vector Borne Disease Control Programme (NVBDCP): Annual Reports, Malaria Statistics, Dengue and Chikungunya Guidelines.

[2] National Vector Borne Disease Control Programme (NVBDCP): Official data and reports on vector-borne diseases in India.

manifesting clinically. Geographic Distribution: Asia and Latin America bear the highest burden of dengue. Dengue fever is a growing public health threat in India, with frequent outbreaks affecting large populations. Incidence: Over 1.3 million cases of dengue were reported in India in 2017, with a significant increase in urban areas. Geographic Distribution: Major outbreaks have occurred in states like Kerala, Delhi, Maharashtra, and Tamil Nadu.

Chikungunya, caused by the chikungunya virus and transmitted by Aedes mosquitoes, has seen large outbreaks since its re-emergence in 2004. Incidence: Chikungunya has caused significant outbreaks in Africa, Asia, Europe, and the Americas. Geographic Distribution: Major outbreaks have occurred in countries like India, Indonesia, Thailand, and several African nations. Chikungunya has caused numerous outbreaks in India, leading to significant public health concerns. Incidence: In 2016, India reported over 64,057 cases of chikungunya, with large outbreaks in Karnataka, Maharashtra, and Delhi. Geographic Distribution: Chikungunya transmission is widespread, affecting both urban and rural areas.

The *Zika virus*, transmitted by Aedes mosquitoes, gained global attention due to its association with microcephaly and other congenital abnormalities. Incidence: The 2015–2016 outbreak affected large parts of Latin America and the Caribbean, with over 500,000 suspected cases. Geographic Distribution: Zika virus transmission has been reported in Africa, the Americas, Asia, and the Pacific.[3] India reported its first cases of Zika virus in 2017, leading to increased surveillance and public health efforts. Incidence: As of 2018, India has reported cases in Gujarat, Rajasthan, and Madhya Pradesh. Geographic Distribution: Zika virus transmission is relatively new in India, with sporadic cases reported in different regions.[4] *Japanese encephalitis (JE)* is caused by the Japanese encephalitis virus, transmitted by Culex mosquitoes. Incidence: JE causes an estimated 68,000 clinical cases globally each year. Geographic Distribution: It is predominantly found in Southeast Asia and the Western Pacific regions. Japanese encephalitis (JE) is endemic in many parts of India, with frequent outbreaks causing high morbidity and mortality. Incidence: India reported over 10,485 cases and 1358 deaths due to JE in 2019. Geographic Distribution: JE is prevalent in states like Assam, Uttar Pradesh, Bihar, and West Bengal. Mortality and Morbidity: JE has a high mortality rate and can lead to severe neurological complications in survivors. *Yellow fever* is caused by the yellow fever virus, transmitted by Aedes and Haemagogus mosquitoes. Incidence: The WHO estimates that there are 200,000 cases of yellow fever annually, with 30,000 deaths. Geographic Distribution: The disease is endemic in tropical areas of Africa and South America. Public Health: Large outbreaks can lead to high mortality and strain public health resources. Vaccination Efforts: The introduction of yellow fever vaccination has significantly reduced the burden in endemic regions.[5]

[3] Centers for Disease Control and Prevention (CDC): Malaria Facts, Dengue Guidelines, Chikungunya Information, Zika Virus Updates.

[4] American Journal of Tropical Medicine and Hygiene: Research Articles and Reviews on Global and Indian Burden of Mosquito-Borne Diseases.

[5] Journal of Clinical Microbiology: Research Articles on Mosquito-Borne Diseases.

10.2 Diagnostic Techniques of Mosquito-Borne Diseases

Mosquito-borne diseases, as discussed above are a major public health concern in India, causing significant morbidity and mortality. These diseases are transmitted to humans by vectors such as mosquitoes, ticks, and fleas. Identifying these diseases accurately is crucial for timely treatment and outbreak control. In the following subsections, we would elaborate various techniques used for identifying mosquito-borne diseases, illustrated with examples from India. While, the focus will be on viral diseases, malaria (a non-viral diseases will also be discussed as a matter of context).

10.2.1 Clinical Diagnosis

Clinical diagnosis is often the first step in identifying vector-borne diseases. It involves a detailed examination of the patient's symptoms and medical history, based on primary symptoms' and patients travel history, evaluation as follows:

- Dengue Fever: In India, dengue fever presents with high fever, severe headache, pain behind the eyes, joint and muscle pain, rash, and mild bleeding. An outbreak in Kerala in 2017 saw a high number of cases with these symptoms.
- Malaria: Common in states like Odisha, Assam, and Jharkhand, malaria symptoms include fever, chills, sweats, headaches, nausea, vomiting, and body aches. During the 2016 outbreak in Odisha, thousands of cases presented these symptoms.[6]
- Japanese Encephalitis: Prevalent in Assam and Uttar Pradesh, symptoms include fever, headache, vomiting, confusion, and seizures. The 2014 outbreak in Assam saw numerous children with these symptoms.[7,8]

Furthermore, assessing recent travel to malaria-endemic regions within India, such as the northeastern states or tribal areas in central India, helps narrow down the diagnosis. Evaluating whether the patient has been in environments with high vector activity, like areas with stagnant water, is crucial. For example, during the chikungunya outbreak in Delhi in 2016, many patients had been exposed to mosquito-prone areas.[9]

[6] World Health Organization (WHO): Comprehensive guidelines on the diagnosis and management of vector-borne diseases.

[7] Indian Council of Medical Research (ICMR): Research Reports on Vector-Borne Diseases in India.

[8] Indian Council of Medical Research (ICMR): Reports and guidelines specific to India's vector-borne diseases.

[9] American Journal of Tropical Medicine and Hygiene: Research articles and reviews on vector-borne diseases, including diagnostic methods.

10.2.2 Laboratory Techniques for Disease Diagnostic

Laboratory techniques are essential for the definitive diagnosis of vector-borne diseases. These techniques range from microscopic examinations to advanced molecular methods.

Microscopy

- Blood Smears: The gold standard for diagnosing malaria.
- Thick Smears: Used to detect the presence of Plasmodium parasites. During malaria outbreaks in Odisha, thick smears have been vital in diagnosing cases.
- Thin Smears: Used to identify the specific species of Plasmodium. In India, *Plasmodium falciparum* and *Plasmodium vivax* are common, and thin smears help differentiate them.
- Giemsa Staining: A special stain used to highlight parasites in blood smears, widely used in Indian laboratories for malaria diagnosis.

Serology

- Enzyme-Linked Immunosorbent Assay (ELISA): Detects antibodies against pathogens such as dengue virus. During the 2015 dengue outbreak in Delhi, ELISA was extensively used to confirm cases.
- Direct ELISA: Detects antigens directly from the pathogen, useful for detecting acute infections.
- Indirect ELISA: Detects antibodies produced in response to the pathogen, useful for retrospective diagnosis.
- Sandwich ELISA: Highly specific, used to detect antigens using two layers of antibodies.
- Rapid Diagnostic Tests (RDTs): Provide quick results and are useful in low-resource settings. For example, during the malaria outbreaks in rural India, RDTs have been critical for quick diagnosis.[10]
  - Lateral Flow Assays: Commonly used for diseases like malaria and dengue. These tests are easy to use and interpret, making them ideal for field use in India.

10.3 Molecular Techniques for Mosquito-Borne Diseases

Molecular laboratory techniques are indispensable for the accurate and early diagnosis of mosquito-borne viral diseases such as dengue, Zika, chikungunya, and West Nile virus. Polymerase chain reaction (PCR) and its variants are among the

[10] American Journal of Tropical Medicine and Hygiene: Research articles and reviews on vector-borne diseases, including diagnostic methods.

most widely used molecular methods for viral detection. Reverse transcription PCR (RT-PCR) is particularly crucial for detecting RNA viruses, as it involves the conversion of viral RNA into complementary DNA (cDNA) before amplification. Real-time PCR (qPCR) further enhances sensitivity and specificity by enabling the quantification of viral load in clinical samples while reducing contamination risks. Loop-mediated isothermal amplification (LAMP) is another valuable technique that offers rapid and cost-effective detection of viral RNA without requiring thermocyclers, making it suitable for field-based applications. Next-generation sequencing (NGS) and metagenomic approaches provide broader insights into viral genetic diversity, co-infections, and emerging mutations, making them essential tools for epidemiological surveillance. Additionally, microarray-based assays facilitate high-throughput screening of multiple pathogens in a single reaction, further improving diagnostic efficiency in outbreak settings. These techniques, when used in combination, enhance the sensitivity and accuracy of mosquito-borne virus detection, aiding in timely disease management and control efforts.

Beyond traditional PCR-based methods, novel molecular diagnostic approaches are emerging to address challenges such as rapid detection, field applicability, and point-of-care testing. CRISPR-based diagnostics, particularly systems like SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) and DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), leverage CRISPR-Cas enzymes for highly specific viral RNA detection, offering rapid, low-cost alternatives with minimal instrumentation requirements. Recombinase polymerase amplification (RPA) is another promising isothermal method that enables rapid and sensitive nucleic acid amplification at constant temperatures, making it ideal for decentralized diagnostic settings. Digital PCR (dPCR) enhances absolute quantification of viral nucleic acids without requiring standard curves, improving precision in low viral load scenarios. Moreover, biosensor-based approaches, including nanoparticle-based assays and electrochemical sensors, are being developed for real-time detection of mosquito-borne viruses with high specificity. Integration of these cutting-edge molecular tools with mobile health (mHealth) platforms and cloud-based data-sharing systems is revolutionizing disease monitoring and outbreak response. While these advancements improve diagnostic capabilities, their widespread implementation requires overcoming challenges related to cost, accessibility, and regulatory approvals, especially in resource-limited regions. A detailed list of primers for conventional molecular detection, serotyping, and species-level genotyping of common arboviruses from human, animal, and field-collected vector specimens is presented in Table 10.1.

Some variants of the PCR commonly used as routine laboratory diagnostics are as below:

- Polymerase Chain Reaction (PCR): Amplifies DNA sequences specific to the pathogen.[11]

[11] Journal of Clinical Microbiology: Research on the latest diagnostic techniques and findings related to infectious diseases.

Table 10.1 Description of primers for conventional molecular detection, serotyping, and genotyping at species level for common arboviruses from biological specimen from human, animal, and field collected vectors

Virus	Primer	Sequence	Amplicon	Genome position
DENV group specific primers (D1 and D2)′ (https://www.who.int/news-room/fact-sheets/detail/vector-borne-disease)	D1 F′	5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′	511	131–161
	D2 R′	5′-TTGCACCAACAGTCAATGTCTTCAGGTTC		616–644
DENV seotype# specific primers (https://www.who.int/news-room/fact-sheets/detail/vector-borne-disease)	D1 F′	5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′	482	
	TS1 R′	5′-CGTCTCAGTGATCCGGGGG-3′		568–586
	TS2 R′	5′-CGCCACAAGGGCCATGAACAG-3′	119	232–252
	TS3 R′	5′-TAACATCATCATGAGACAGAGC-3′	290	400–421
	TS4 R′	5′-CTCTGTTGTCTTAAACAAGAGA-3′	392	506–527
CHIKV detection primer (Schmaljohn and McClain 1996)	NspF′	5′-TAGAGCAGGAAATTGATCCC-3′	354 bp	228–247
	NspR′	5′-CTTTAATCGCCTGGTGGTAT-3′		581–562
CHIKV E-gene typing primer primer (Weaver 2013)	E1-F	5′-ACAAAACCGTCATCCCGTCTC-3′	992 bp	10,145–10,165
	E1-R	5′-TGACTATGTGGTCCTTCGGAGG-3′		11,137–11,158
CHIKV detection and genotyping primer (Shope and Meegan 1997; https://www.cdc.gov/dengue/)	CHIK/E1-S	5′-TACCCATTCATGTGGGGC-3′	294 bp	10,246–10,263
	CHIK/E1-c	5′-GCCTTTGTACACCACGATT-3′		10,539–10,521
CHIKV detection primer (Dhara et al. 2013)	CHIK F′	5′-ACGCAGTTGAGCGAAGCAC-3′	205 bp	10,294–10,312
	CHIK R′	5′-CTGAAGACATTGGCCCCAC-3′		10,498–10,480
West Nile virus (Rocklöv and Dubrow 2020; Debnath et al. 2021)	WNF	5′-TGGATTTGGTTCTCGAAGG-3′	200 bp	1028–1046
	WNR	5′-GGTCAGCACGTTTGTCATT-3′		1228–1210
	WN233	TTGTGTTGGCTCTCTTGGCGTTCTT	408 bp	233–257
	WN640c	CAGCCGACAGCACTGGACATTCATA		640–616
Japanese encephalitis virus (https://nvbdcp.gov.in/index4.php?lang=1&level=0&linkid=431&lid=3715)	JEV-prMf	5′- CGTTCTTCAAGTTTACAGCATTAGC -3′	675 bp	251–275
	JEV-prMr	5′- CCYRTGTTYCTGCCAAGCATCCAMCC -3′		901–925
	JEV-Ef	5′- TGYTGGTCGCTCCGGCTTA-3′	1580 bp	955–973
	JEV-Er	5′- AAGATGCCACTTCCACAYCTC-3′		2516–2536

(continued)

Table 10.1 (continued)

Virus	Primer	Sequence	Amplicon	Genome position
JEV detection and genotyping (Yadav et al. 2019; Integrated Disease Surveillance Program (IDSP) 2018)	JEV-F	5′- GCAGAAAGCAAAACAAAAGAG-3′	350 bp	
	JEV-R	5′- ACGGATCTCCTGCTTCGCTTG-3′		
Zika virus (https://scroll.in/article/1010555/inside-uttarpradeshszika-outbreak-can-indias-most-populous-state-contain-the-virus-spread)	ZIKV F	5′-CCGCTGCCCAACACAAG-3′	76 bp	1086–1102
	ZIKV R	5′-CCACTAACGTTCTTTTGCAGACAT-3′		1162–1139
Chandipura virus (Yadav et al. 2022)	CHPNDGF3	5′ TGATTCCTACATGCCCTATCT 3′	257 bp	821–841
	CHPNDGR3	5′ GAACTTCTTCCCGTTAAGCACG 3′		1078–1057
	CHPNDGF4	5′ TCCACGAAGTCTCCTTACTCT 3′	198 bp	863–883
	CHPNDGR4	5′ GCACGAATCTCTGCTCCAGCT3′		1061–1041

  - Nested PCR: Increases sensitivity by using two sets of primers. Used in India for detecting low-level malaria infections.
  - Multiplex PCR: Detects multiple pathogens simultaneously. In India, this technique is used to differentiate between dengue, chikungunya, and Zika viruses during outbreaks.

- Real-Time PCR (qPCR): Quantifies the amount of pathogen DNA/RNA in a sample, providing information about the severity of infection. This technique was used during the 2018 Zika virus outbreak in Rajasthan.
- Reverse Transcription PCR (RT-PCR): Converts RNA into DNA before amplification. Used for RNA viruses like Zika and West Nile virus. RT-PCR was crucial in identifying Zika virus cases in Jaipur.

PCR protocols for detecting mosquito-borne viruses vary depending on the target virus, sample type, and diagnostic objectives. Reverse transcription PCR (RT-PCR) is commonly used for RNA viruses like dengue, Zika, and chikungunya, requiring an initial reverse transcription step to convert RNA into complementary DNA (cDNA) before amplification. Real-time PCR (qPCR) further refines this process by enabling quantitative detection with fluorescence-based probes, improving sensitivity and specificity while reducing contamination risks. Nested PCR, which involves two successive rounds of amplification, enhances specificity for low-viral-load samples but increases the risk of contamination due to multiple handling steps. Multiplex PCR allows simultaneous detection of multiple arboviruses in a single reaction by using virus-specific primer sets, optimizing efficiency in outbreak scenarios. Digital PCR (dPCR) provides absolute quantification of viral nucleic acids without reliance on standard curves, making it suitable for detecting low-copy viral RNA. Isothermal amplification methods like loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) offer rapid, field-deployable alternatives to conventional PCR, eliminating the need for thermal cycling. Variations in PCR protocols also arise based on the choice of polymerases, primer design, cycling conditions, and sample extraction methods, all of which influence detection sensitivity, specificity, and applicability in field surveillance or clinical diagnostics. Table 10.2 and 10.3 enlist some common PCR protocols and primers for real-time PCR analysis of viral diseases respectively.

10.4 Challenges of Diagnosis and Entomological Surveillance

Entomological surveillance plays a vital role in complementing molecular diagnostics by providing critical insights into vector ecology, distribution, and population dynamics. Vector identification is a key component, as accurately determining the species responsible for transmission helps in understanding disease ecology and devising targeted control measures. Various entomological surveys aid in the systematic collection of mosquito vectors, employing diverse methods such as light traps, bait traps, and manual collection. Light traps, which attract and capture mosquitoes using artificial light, have been instrumental in Karnataka for studying

Table 10.2 Viral-families/genus-based consensus PCR protocol for laboratory analyses of human and animal samples

Specimens	Human proposed test	Consensus (c) PCR	Virus family	Target gene	Amplicon	Primer sequences (5′–3′)
Oral/Nas al Swab, Blood, CSF, and stool samples (Tandel et al. 2019)	Sanchez-Seco et al. (2001)	Alphavirus consensus PCR (twostep, nested PCR)	*Alphaviruses genus*	Alphavirus NSP4 gene sequence	*PCR1-481 bp*	F′ = 5′-GAYGCITAYYTIGAYATGGTIGAIGG 3′ R′ = 5′-KYTCYTCIGTRTGYTTIGTICCIGG -3
					PCR2-195 bp	F′ = 5′-GIAAYTGYAAYGTIACICARATG′-3 R′ = 5′-GCRAAIARIGCIGCIGCYTYIGGICC -3′
Oral/Nas al Swab, Blood, CSF, and stool samples (Mishra et al. 2011)	de Morais et al. (2005)	Pan *alfavirus* primers	*Alphaviruses genus*	Alphavirus NSP1 gene sequence	*PCR 430 bp*	F = 5′-YAGAGCDTTTTCGCAYSTRGCHW-3′ R = 5′-ACATRAANKGNGTRTCRAANCCDAYCC-3′
Oral/Nas al swab, Blood, CSF, and stool samples (Mourya et al. 2012)	Moureau et al. (2007)	Flaviviruses consensus PCR: Onestep reverse transcripton (RT) based conventional PCR assay	*Flaviviridae*	NS5 gene	*PCR amplicon size:270 bp*	F′ = 5′-TGYRBTTAYAACATGATGGG-3′ R′ = 5′-GTGTCCCAICCNGCNGTRTC-3′

Oral/Nas al swab, Blood, CSF, and Stool samples	de Morais et al. (2005)	Pan *flavivirus* primers Onestep reverse transcripton (R T) based conventional PCR assay	*Flaviviridae*	RNAdependen t RNA polymerase (RdRp) domain, located at the carboxy terminus of NS5	*>900 bp*	F′ = 5′-TACAACATGATGGGAAAGCGAGAGAAAAA-3′ R′ = 5′-GTGTCCCAGCCGGCGGTGTCATCAGC-3′
Oral/Nasal swab, Blood, CSF, and Stool samples (Mourya et al. 2015)	Bourhy et al. (2005)	*Rhabdoviruses* semi-nested PCR	*Rhabdoviridae*	L *gene*	*PCR1-460 bp*	F′ = 5′-CCADMCBTTTTGYCKYARRCCTTC-3′ R′ = 5′-RAAGGYAGRTTTTTYKCDYTRATG-3′
					PCR2-260 bp	F′ = 5′-CCADMCBTTTTGYCKYARRCCTTC −3′ R′ = 5′-AARTGGAAYAAYCAYCARMG-3′

Table 10.3 Description of primers and probes for Real time RT-PCR detection of common arboviruses from human, livestock/domestic animals, and field collected vectors

Virus	Primer	Sequence	Genome position
Dengue virus (Mourya and Yadav 2013)	D1F	CAAAAGGAAGTCGYGCAATA	8936–8955
	D1R	CTGAGTGAATTCTCTCTGCTRAAC	9023–9047
	D1-probe	CATGTGGYTGGGAGCRCGC	8961–8979
	D2F	CAGGCTATGGCACYGTCACGAT	1426–1447
	D2R	CCATYTGCAGCARCACCATCTC	1482–1504
	D2-probe	CTCYCCRAGAACGGGCCTCGACTTCAA	1454–1480
	D3F	GGACTRGACACACGCACCCA	701–720
	D3R	CATGTCTCTACCTTCTCGACTTGYCT	749–775
	D3-probe	ACCTGGATGTCGGCTGAAGGAGCTTG	722–747
	D4F	TTGTCCTAATGATGCTRGTCG	884–904
	D4R	TCCACCYGAGACTCCTTCCA	953–973
	D4-probe	TYCCTACYCCTACGCATCGCATTCCG	939–965
Chikungunya (Tandale et al. 2015)	CHIK F	GAGCATACGGTTACGCAGATAG	3855–3876
	CHIK R	TACTGGTGATACATGGTGGTTTC	3957–3979
	CHKV-probe	ACGAGTAATCTGCGTACTGGGACGTA-FAM	3886–3915
West Nile virus (Murhekar et al. 2015)	WNV-F	TCAGCGATCTCTCCACCAAAG	1160–1180
	WNV-R	GGGTCAGCACGTTTGTCATTG	1209–1229
	WNV-probe	TGCCCGACCATGGGAGAAGCTC-FAM	1186–1207
Japanese encephalitis virus (Awate et al. 2016)	JE-F	GCCACCCAGGAGGTCCTT	3588–3605
	JE-R	CCCCAAAACCGCAGGAAT	3633–3650
	JE-probe	CAAGAGGTGGACGGCC-FAM	3608–3623

Zika virus (https://scroll.in/article/1010555/inside-uttarpradeshszika-outbreak-can-indias-most-populous-state-contain-the-virus-spread)	ZIKV F	CCGCTGCCCAACACAAG	1086–1102
	ZIKV R	CCACTAACGTTCTTTTGCAGACAT	1162–1139
	ZIKV-probe	AGCCTACCTTGACAAGCAGTCAGACACTCAAFAM	1107–1137
Chandipura virus (Yadav et al. 2022)	PF1	TTTAATCGACATGGGAGCAATTG	1953–1975
	PR1	TAAGGTGGGTCAGACGGAGAGA	2021–2000
	PP TaqMan MGB-probe	VIC-AGAATTCATCCTGGCAGCT-NFQ	1980–1998

Anopheles mosquito populations, which are responsible for malaria transmission. Bait traps, using CO_2 or other chemical attractants, have been successfully utilized in Maharashtra to monitor Aedes mosquitoes, the primary vectors of dengue and chikungunya. Manual collection, involving the direct capture of vectors from their habitats using nets or aspirators, remains a critical method in rural India, particularly for sandflies responsible for kala-azar and Chandipura virus transmission. Identification of these vectors is crucial and is conducted through both morphological and molecular techniques. Traditional morphological identification, using taxonomic keys, remains widely practiced in India for classifying mosquito and sandfly species based on their physical characteristics. However, molecular identification, involving PCR and sequencing techniques, provides higher accuracy and is particularly useful for distinguishing morphologically similar species, such as Aedes aegypti and Aedes albopictus. The integration of molecular identification with entomological surveillance enhances diagnostic precision by confirming the presence of disease-carrying vectors in endemic regions.

Epidemiological surveillance, closely linked with entomological monitoring, is essential for predicting and controlling mosquito-borne viral outbreaks. By systematically tracking disease incidence and vector populations, epidemiological data aids in risk assessment and outbreak preparedness. Geographic Information Systems (GIS) play a crucial role in mapping disease incidence and visualizing the distribution of both vectors and infections. In India, GIS has been effectively used to map malaria prevalence in tribal regions, helping in targeted intervention strategies. Spatial analysis further refines this process by identifying high-risk areas and potential hotspots, as seen in Delhi for dengue surveillance. Remote sensing, another powerful tool, leverages satellite imagery to monitor environmental factors influencing vector populations, such as temperature and vegetation density. This approach has been successfully applied in Kerala to assess regions at risk for Japanese encephalitis. Sentinel surveillance, which involves selected healthcare facilities or communities acting as reporting sites, contributes valuable real-time data on disease incidence. In Tamil Nadu, this method has been effectively utilized for dengue monitoring, providing early warning signals of outbreaks. Trend analysis, another essential aspect of epidemiological surveillance, enables health authorities to track changes in disease patterns over time. For instance, trend analysis played a crucial role in the early detection of chikungunya outbreaks in Karnataka, allowing for timely public health interventions. By integrating entomological and epidemiological surveillance with molecular diagnostics, health authorities can develop more effective vector control programs and improve early disease detection, ultimately mitigating the impact of mosquito-borne viral diseases.

10.5 Future Challenges and Needs in Molecular Diagnosis

The future of molecular diagnostics for mosquito-borne viral diseases is set to advance significantly with the development of more rapid, field-deployable, and cost-effective technologies. Traditional molecular diagnostic methods, such as

RT-PCR and sequencing, though highly accurate, often require well-equipped laboratories, trained personnel, and significant infrastructure. To overcome these limitations, emerging technologies are focusing on the development of point-of-care (POC) diagnostic tools that can be used in remote and resource-limited settings. Lateral flow assays, which provide results within minutes, have already been successfully implemented for malaria, dengue, and chikungunya detection. In rural Bihar, these rapid tests have played a crucial role in timely malaria diagnosis, allowing for immediate treatment initiation. Similarly, portable PCR devices, which offer the sensitivity and specificity of laboratory-based PCR testing in a compact, battery-operated format, are transforming field diagnostics. These devices were instrumental during the 2018 Zika virus outbreak in Rajasthan, enabling on-site detection and helping to contain the spread of the virus. The integration of isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP) with these portable devices is expected to further enhance field-based molecular diagnostics by eliminating the need for thermal cycling, thereby reducing operational complexity and turnaround time.

Despite these advancements, emerging threats such as climate change, urbanization, and increased global travel continue to complicate mosquito-borne disease management. Climate change has expanded the geographical range of vector populations, leading to the emergence of arboviral diseases in previously unaffected regions. Urbanization and environmental degradation have created new breeding grounds for mosquitoes, while increased international travel has facilitated the spread of viruses across borders. To mitigate these threats, a multipronged approach is required, integrating enhanced molecular diagnostics with robust epidemiological surveillance and vector control strategies. The use of Geographic Information Systems (GIS) and artificial intelligence (AI) for real-time disease mapping and predictive modeling can help in early outbreak detection and response planning. Additionally, strengthening public health infrastructure, improving data-sharing mechanisms, and fostering international collaborations will be essential in managing the global burden of mosquito-borne diseases. The development and widespread deployment of cost-effective, user-friendly, and portable molecular diagnostic devices will play a pivotal role in early detection and rapid response, ultimately reducing the impact of these diseases on public health.

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Chapter 11
Molecular Diagnosis of Enterovirus: Concepts and Challenges

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Abstract Enterovirus includes a diverse group of RNA-viruses that is part of the family of picornaviridae. Even though these viruses are distributed worldwide, there are some unpleasant illnesses they cause such as, minor respiratory tract infections and severe neurological diseases. The reason for this is the presence of extensive variability in the enterovirus genome and the ability of the like virus to spread without culture symptoms. Enteroviruses have been diagnosed since a long time using serological approaches but often encounter the drawbacks such as time-consuming tests, low-specificity, and low-sensitivity. The invention of polymerase chain reaction and next generation sequencing has fundamentally changed the approach to detection and typing of enteroviruses. They combine very high specificity together with very high sensitivity and this allows detecting of more than 10 enteroviruses including those that have a high mutation rate as well as those with low viral loads. However, co-existing infections are a significant problem because these diseases exhibit significant genetic diversity. This chapter aims to discuss about the fundamentals of enterovirus molecular diagnostics, highlighting important methods, their advantages, and their drawbacks. It also addresses the main difficulties in molecular diagnosis of enteroviruses and describes new strategies to get beyond these barriers.

Keywords Enterovirus · Molecular-diagnosis · RNA-virus · CRISPR · Serological-assays

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11.1 Introduction

Enteroviruses represent a highly diverse genus of tiny viruses with icosahedral geometry and non-enveloped nature. Enteroviruses belong to the picornaviridae family; they have single positive strand RNA genome as genetic material. Enterovirus virions are more stable and they are highly resistance to the acidic environments such as pH 3.0 and below. This characteristic of enterovirus makes them survive and replicate in gastrointestinal tract and faecal-oral route and this is their primary route of transmission (Laitinen et al. 2016). The enterovirus genus is further divided into species A to L based on their sequence diversity, within which, the human enterovirus species (A to D) cause infection to millions of people worldwide every year (Chen et al. 2020). Three poliovirus serotypes (poliovirus 1–3) are found in enterovirus C species and they are commonly referred as non-polio enteroviruses (Simmonds et al. 2020). Enteroviruses are also divided into subspecies based on their hyper-variable protein VP1 and they are called as "types". As of 2025, 326 enterovirus types have been identified in the 15 different species. This classification includes 168 different rhinovirus types, 3 poliovirus types, and 103 enterovirus types, these enterovirus types infect humans and lead to large outbreaks (Brown et al. 2020). For the designation of novel enterovirus types, the prefix EV or RV has been used, followed by the species letter and a type number. Some enterovirus kinds have had distinct names for historical reasons. All echoviruses are members of the enterovirus B species, they are abbreviated with the prefix "E" (for instance, E30) even though they are not members of the enterovirus E species. Some coxsackievirus (CV) types, identified by the CV prefix, can be categorized as EV-A, EV-B, or EV-C species based on the characteristics of their VP1 sequence (Brown et al. 2020).

11.1.1 Pathogenesis of Enterovirus

The majority of enteroviruses cause illness by infecting the gastrointestinal tract's epithelial linings, which enables the virus to travel to other parts of the body (Wells et al. 2022). The initiation of enterovirus replication cycle involves adhesion and binding of the viral particles to the cellular receptor of the host cells, then receptor mediated internalization takes place. Later on the viral genome is released into the host cell and translated into single polyprotein. The single polyprotein processed to mediate the non-structural and structural proteins. The newly produced viral genome is converted into new viral particles that are released from cells through lysis or budding (Ayyub et al. 2024). Enteroviruses are the most common etiological agent for the aseptic meningitis and encephalitis which are inflammation of meninges on the brain and spinal cord or the brain only. Initially the virus spreads through the aerosols or the faecal particles and enter into the gastrointestinal tract or respiratory tract. The virus replicate there and travel to the central nervous system where they cause meningitis or other neurological problems. Responding to the infection, the

immune system increases the production of the CD8+/T cells, monocytes, and neutrophils (Lee et al. 2012).

The enteroviruses are highly contagious and they frequently cause infection asymptomatically but human enteroviruses exhibit a variety of symptoms such as fever, vomiting, diarrhoea, headache, sore throat, respiratory illness. Sometimes human enteroviruses cause severe clinical disorders such as aseptic meningitis and severe encephalitis because they are neurotropic pathogen (Hasbun et al. 2017; Balada-Llasat et al. 2019). Furthermore, enterovirus cause hand, foot, and mouth disease (HFMD), myocarditis, conjunctivitis, and gastrointestinal tract diseases (Xie et al. 2024). The severe cases of the HFMD associated with enterovirus result in death (Yi et al. 2017).

11.1.2 Epidemiology of Human Enteroviruses

The spreading dynamics of enteroviruses are divided into two patterns, they are endemic and epidemic. The epidemic pattern was observed when the expansions and contractions that occur on multi-year cycle pattern or an annual pattern. The endemic pattern was observed when one type spread in low level for the whole year and it may or may not be detectable. Sometimes new strains or types such as EV-D68 and EV- A71 can start spreading in the endemic pattern and turn to epidemic pattern later. On the epidemic phase, the diversity of virus significantly increase like EV-D68 have emerged multiple clades (A, B, and C) within a 13-year period (Brown et al. 2020). Enterovirus has been predominant cause of encephalitis/meningitis in the USA (Hasbun et al. 2017; Balada-Llasat et al. 2019). Enterovirus A species Enterovirus A-71 is the major etiological agent for the HFMD, enterovirus A-71 has a wide range of diversity because it contains 25 serotypes. The children below or at the age of five majorly get affected by the enterovirus A-71. Enterovirus A-71 cause neurological complications such as acute flaccid paralysis, aseptic meningitis, neurogenic pulmonary edema, brainstem encephalitis, and delayed neurodevelopment and reduced cognitive function (Yuan et al. 2018). The enterovirus A71 became endemic in Asian-pacific region from 1990 and in every 3–4 years it causes large outbreak (Puenpa et al. 2019).

Enterovirus B contains 63 genotypes, and major etiological agents such as echoviruses (E3, E6, E9, E11, E18, E30) and coxasackiviruses B (CVB1–5). the enterovirus B co circulates with enterovirus A and enterovirus D and causes notable neurological disease worldwide. Enterovirus B mainly targets the CNS, causing encephalitis and aseptic meningitis. Genotypes like E30 and CVB1–5 cause infection mainly in infants. In USA, enterovirus B is a predominant etiological agent for the neurological disease cases commonly identified in cerebrospinal fluid. In Europe, it is the most common species among enteroviral infections and majorly associated with neurological manifestations. In Asia, enterovirus A holds major record for causing HFMD outbreaks, sporadic cases but other complications might be caused by the enterovirus B. The widespread occurrence of enterovirus B and

their diversity make them important in enteroviral research and surveillance (Xie et al. 2024). The non-polio enterovirus C is uncommon but the specific strain CV-A24 has caused widespread outbreaks of conjunctivitis which is the inflammation of the outer membrane of the eyelid (Brown et al. 2020). The CV-A24 spreads through contact with eye secretions, it's majorly observed in the tropics during rainy season. It has caused outbreaks in several countries such as French, China, Guiana, India, Western Africa, and Brazil (Medina et al. 2016; Zhang et al. 2016; Enfissi et al. 2017; Burr et al. 2017). Other enterovirus C species can cause various diseases such as HFMD, AFP, encephalitis, respiratory diseases, and meningitis (Brown et al. 2020).

Enterovirus D68 caused major outbreak of serious respiratory illness and acute flaccid myelitis and polio like paralysis in different countries at 2014 (Holm-Hansen et al. 2016). The enterovirus D68 spread through the infected surface and respiratory secretions. In Europe and North America from 2014 to 2018 the outbreaks occurred in every 2 years. After COVID 19 interruption, the enterovirus D68 again occurred in the 2021 and 2022 (Benschop et al. 2021). The genetically heterogeneous enterovirus D68 have four main clades, A-D and clade B3 are frequently linked to current out breaks and AFM patients. AFM is a rare but severe disease. Children are disproportionately affected by this virus, most common symptom is respiratory tract illness. Studies conducted worldwide show that exposure rises with age and reaches almost universal levels in adults, indicating a high likelihood of reinfections or cross-reactivity with other enteroviruses (Holm-Hansen et al. 2016). AFM is closely associated with enterovirus D68, which mostly cause respiratory conditions like bronchiolitis and severe respiratory illness. The virus is airborne and contact based and spreads through respiratory secretions and contact with contaminated surfaces. In 2014 and 2018, major enterovirus D68 outbreak happened in the USA. In 2014, the outbreak alone resulted in 277 cases of AFM and 1542 instances of severe respiratory illness, in 2022 the enterovirus D68 occurred again. The most frequent symptom of enterovirus D68 is respiratory illness that was highly reported in Europe than USA (Xie et al. 2024). In that same year, enterovirus D68 caused over 1300 confirmed cases, predominantly among children which resulted in five deaths. The outbreak of enterovirus also associated with several cases of polio like syndromes (Christy and Messacar 2019). From that enterovirus D68 emerged as epidemic in USA and Europe (Christy and Messacar 2019; Gilrane et al. 2020). The sero-epidemiological studies revealed that sustained and high frequency of enterovirus D68 infection in Chinese population; it affected the various age groups of people. The seroprevalence rate was positively correlated with 1-year old children to 15-year old children and negatively correlated with infants who are 1 month old (Xiang et al. 2017; Sun et al. 2018).

In other countries, the B3 subclade strains of enterovirus D68 emerged as most important etiological agent that causes paediatric lung disease and AFM which requires supplemental oxygen. China reported distinct enterovirus D68 genotype that prevailed from 2011 to 2018 and it showed recombination characteristic (Holm-Hansen et al. 2016; González-Sanz et al. 2019; Messacar et al. 2020). In early

2000s, the closely associated enterovirus types like enterovirus- B93, enterovirus-D94, and enterovirus- D111 caused AFM outbreaks in both Egypt and Congo (Jartti et al. 2024). Enterovirus- B93 strain has undergone recombination with enterovirus-B107, the enterovirus- B93 exhibited prognosticative virulence, temperature resistance and it has the potential for a large scale outbreak (Zhang et al. 2020). There are 15 non-polio enterovirus (NPEV) species and 7 out of that (EV A-D and RV A-C) infect humans. Worldwide NPEV is a common human pathogen that frequently affects the children (Simmonds et al. 2020). The outbreak of NPEV holds a risk to human health. Sixty-six different NPEV types were identified in 24 EAA/EU countries in 2015 and 2017. This leaded to 68 deaths, 77 case of paralysis, and over 3000 neurological infections (Bubba et al. 2020).

The control of enterovirus spreading is depending on the early and proper detection in appropriately collected specimens. The rapid and early diagnosis of enterovirus is essential for preventing viral culture from spreading to community and individual (Van Leer-Buter et al. 2016). Compared to the traditional methods like viral culture and serology, the molecular diagnostic techniques revolutionized the detection of enteroviruses. Molecular diagnostic techniques have huge advantages like high sensitivity and specificity, and they can detect enterovirus rapidly. Molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) enable the detection and genotyping of enteroviruses directly from the clinical specimens (Harvala et al. 2018). The advantages of these molecular techniques make them invaluable for clinical diagnostics and epidemiological surveillance. Even developed molecular methods with increased sensitivity and speed couldn't detect certain species of enterovirus such as EV-C105, EV-C109, and EV-D68 (Harvala et al. 2018). The large outbreaks of enterovirus species such as EV-A71 in Asia, EV-D68 in USA and Europe leaded to develop the type specific RT-PCR assays which allowed the rapid and effective identification (Poelman et al. 2015; Harvala et al. 2018).

However, there are several obstacles in the way of enterovirus molecular diagnostics. Diagnostic gaps may result from the difficulty in designing universal primers and probes due to the great genomic variation both within and between enterovirus species. Furthermore, the numerous recombination events that enteroviruses display make sequence-based identification and genotyping even more challenging. RNA viruses have a high rate of mutation, mismatches in primer or probe binding sites may potentially cause misleading negative results. In co-infections involving many enterovirus strains or other pathogens, or in patients with no symptoms, the interpretation of molecular diagnostic data might be challenging.

This chapter aims to discuss about the fundamentals of enterovirus molecular diagnostics, highlighting important methods, their advantages, and their drawbacks. In order to ensure precise detection and monitoring of these globally important infections, it also addresses the main difficulties in molecular diagnosis of enteroviruses and describes new strategies to get beyond these barriers. Diagnosis of enterovirus infections can be detected both in conventional non-molecular diagnostic methods and molecular diagnostic approaches.

11.2 Conventional Non-molecular Diagnosis of Enterovirus

Cell culture, serological tests, and other direct detection methods are coming under the category of conventional non-molecular diagnostic methods to detect enterovirus infection.

11.2.1 Isolation of Virus in Cell Culture

For decades that the virus has been inoculated and cultured in cell culture often referred as the "gold standard" for detecting viable viruses. To isolate enteroviruses, clinical specimens such as stool, throat swabs, cerebrospinal fluid, tissue biopsies, and nasopharyngeal aspirates are inoculated onto susceptible cell lines. Specimens are usually prepared by centrifugation and filtered supernatant to remove bacteria and other contaminants. Commonly used cell lines to isolate enteroviruses include Human Rhabdomyosarcoma (RD) cells, HeLa cells, and Vero cells. The processed specimens are inoculated onto monolayer of the selected cell lines under 33–37 °C of incubation. Cytopathic effect appear over a period of days to weeks (within 3–14 days), indirect evidence of viral presence can be observed under a microscope with characterization of some morphological changes such as cell rounding, cell lysis, or syncytium formation. Without any prior knowledge, a wide range of enterovirus serotypes can be detected and also cell culture derived viruses are indispensable for vaccine production, which are the major advantages of cell culture for enterovirus isolation. Unsuitable for rapid diagnosis, sensitivity issues, lack of universal cell lines, false positives due to contaminants by other microbes are the major challenges in cell lines.

Diagnosing enterovirus infections using conventional non-molecular approaches presents several challenges. Clinical examination, serological tests, and imaging are the conventional methods of diagnosis, which can be used to detect these infections accurately. The non-molecular approach to diagnosing enterovirus infections involves isolating the virus in cell cultures. Viral replication is detected using this method in laboratory cell cultures. Therefore, throat swabs, stool, and CSF can be used for virus isolation. Virus culture is always time-consuming because it takes several days to weeks to yield results. The delay in results presents an important issue, particularly in cases where rapid diagnosis and treatment are critical. In a few cases, the viral load in clinical samples may be too low for successful isolation in culture, resulting in false negatives. Furthermore, enterovirus strains may not grow well in standard cell lines, limiting the utility of viral cultures.

11.2.2 Imaging

Conventional imaging techniques such as CT or MRI help to identify problems like brain swelling or myocarditis in severe enterovirus infection, but they are not useful for detecting the virus. While imaging can detect structural changes, it cannot identify the viral cause or detect the infection during an early stage. When viral meningitis or encephalitis is caused by enterovirus, imaging may identify complications too late, after the infection has significantly progressed, making early detection and treatment more challenging.

11.2.3 Serological Tests

Serological tests detect antibodies such as IgM and IgG in the patient's blood, which show an immune response to the enterovirus. These tests can provide indirect evidence of recent or past infection. Especially in the early stages of infection, serological tests for enteroviruses may not provide immediate result. During the early stages, antibody levels might still be too low to detect. Serological tests may also suffer from cross-reactivity because multiple enterovirus serotypes exist, which may give rise to false positives or negatives. This can make it difficult to absolutely identifying the infecting enterovirus strain and cannot always reflect the current infection status. Serological tests might not always differentiate between a recent infection and past exposure, complicating result interpretation particularly in individuals with prior enterovirus infections. Types of serological tests for enterovirus include:

- *Neutralization assay*: This is considered a gold standard method for confirming an enterovirus infection. It investigates the capacity of the patient's serum to neutralize the virus in vitro, which evidences the existence of certain antibodies. This test does not seem to be simple and fast and is not widely used in the clinic.
- *Hemagglutination inhibition*: This test is mainly done for viruses like influenza and in certain occasions, to enterovirus as well. It assesses the capacity of antibodies in preventing the virus from causing agglutination [hemagglutination] of red blood cells.
- *Enzyme linked immunosorbent assay [ELISA]*: IgM or IgG enterovirus antibodies detection in the blood which takes a form of ELISA is used as an indirect marker of infection. IgM antibodies are linked with acute infection while IgG antibodies indicate an old infection or an already developed immunity.
- *Complement fixation test*: This test's purpose is to identify the virus's antibodies by observing the fixation of the complement proteins that is a sign of the body's immune system being activated.

11.2.4 Invasive Procedures

In the enteroviral meningitis or encephalitides suspects, a lumbar puncture in these patients is indicated in order to retrieve the cerebrospinal fluid. Although it is classified as an invasive approach which may be uncomfortable to patients and has possible side effects such as bleeding or infection. It may be quite difficult to do in some patients like infants or edge-of-life patients. It examines the ability of patient serum to neutralize the virus in vitro, thus confirming the presence of specific antibodies. This test may be time-consuming and is not commonly used in clinical practice. Whereas CSF analysis can provide valuable information, it may not always yield definitive result for enteroviral infections.

11.2.5 Antigen Detection Assays

Antigen detection assay involves identifying specific viral proteins in clinical samples. These methods do not require live virus propagation and offer a faster alternative to viral culture. Some commonly used antigen-detection assays are as below:

Immunofluorescence Assay: This technique uses fluorescently labelled antibodies that particularly bind to enteroviral antigens in patient samples. Fluorescence microscopy is then utilized to identify the antibody virus complex that has been bound together. While this approach is quick and straightforward to implement, it does depend on the expertise of the personnel involved and may not be as sensitive, in some cases.

Enzyme Linked Immunosorbent Assay (ELISA): This approach is commonly employed to identify proteins, in samples taken from patients including fluid or throat swabs offering a quicker diagnostic alternative to viral isolation and proving suitable, for clinical application.

Immunohistochemistry (IHC): This method is utilized to identify proteins, in tissue samples such, as biopsies or autopsy specimens by using antibodies that specifically attach to enterovirus proteins and enabling the visualization of antigens through a microscope.

Antigen detection assays are particularly useful in cases where the virus is present in very low quantities or in specific tissue types, such as viral meningitis or myocarditis. Although it requires invasive tissue sampling and it may be difficult to perform without access to specialized laboratory resources.

In summary of non-molecular methods of enterovirus detection, we can comment that these methods are valuable tools for initial detection, epidemiological studies, and identification of virus strains, but they have certain limitation in sensitivity, specificity, and turnaround time, this is the reason molecular techniques, such as PCR have become widespread for quick and precise diagnosis as well as gold-standard for reference laboratories. In spite of this, non molecular approaches

remain important, mainly when molecular resources are unavailable such as in field setting or in limited healthcare resource settings.

11.3 Molecular Diagnosis of Enterovirus

The control of enterovirus spreading is depending on the early and proper detection in appropriately collected specimens. The rapid and early diagnosis of enterovirus is essential for preventing viral culture from spreading to community and individual (Van Leer-Buter et al. 2016). Compared to the traditional methods like viral culture and serology, the molecular diagnostic techniques revolutionized the detection of enteroviruses. Molecular diagnostic techniques have huge advantages like high sensitivity and specificity, and they can detect enterovirus rapidly. Molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) enable the detection and genotyping of enteroviruses directly from the clinical specimens. The advantages of these molecular techniques make them invaluable for clinical diagnostics and epidemiological surveillance. Even developed molecular methods with increased sensitivity and speed couldn't detect certain species of enterovirus such as EV-C105, EV-C109, and EV-D68 (Harvala et al. 2018). The large outbreaks of enterovirus species such as EV-A71 in Asia, EV-D68 in USA and Europe leaded to develop the type specific RT-PCR assays which allowed the rapid and effective identification (Poelman et al. 2015; Harvala et al. 2018). However, there are several obstacles in the way of enterovirus molecular diagnostics. Diagnostic gaps may result from the difficulty in designing universal primers and probes due to the great genomic variation both within and between enterovirus species. Furthermore, the numerous recombination events that enteroviruses display make sequence-based identification and genotyping even more challenging. For RNA viruses, this comes handy since they have high mutation frequency but it also means that mismatches in primers or probes sites can lead to negative results that are false. Moreover, the context of patient with asymptomatic carriers or multiple enterovirus strains or other pathogens co infection might complicate the minimalist view of an interpretation for such a data. It is needless to say that molecular techniques for diagnosis of enterovirus infections should be included into practice because of their sensitivity, specificity, and quick response. In the following sections, the main molecular methods that are commonly used in the diagnosis of enteroviruses along with their pros and cons.

11.3.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the most common nucleic acid amplification technique (NAAT) test used to identify the EV genome in cell cultures, clinical specimens, and biopsy tissues (Table 11.1). These tests are very sensitive and

Table 11.1 Summary of PCR-based approaches used for the molecular diagnosis of enterovirus, along with the corresponding kit names and manufacturers

Kit name	Manufacturer	Detection method	Target region	Reference
Hi-PCR® Enterovirus Probe PCR Kit	HiMedia Laboratories	Real-time PCR	5′ UTR	HiMediaLaboratories
GeneProof Enterovirus PCR Kit	GeneProof	Real-time PCR	5′ UTR	GeneProof
ViroReal® Kit Enterovirus	Ingenetix GmbH	RT real-time PCR	5′ UTR	Ingenetix GmbH
RealStar® Enterovirus RT-PCR Kit 1.0	Altona Diagnostics GmbH	RT real-time PCR	Not specified	altona Diagnostics
TaqPath™ Menu | GeneProof™ Enterovirus PCR Kit	Thermo Fisher Scientific	Real-time PCR	5′ UTR	Thermo FisherScientific

achieve 100% accuracy in the absence of laboratory cross-examination. These tests are mainly based on viral RNA, which target the conserved 5′ NTR region in EV. The process of Polymerase Chain Reaction (PCR) is a common molecular wiping technique that enables the amplification of specific DNA or RNA sequences containing enteroviral RNA. This technique aims at specific portions of the enterovirus genome and is important for the diagnosis of enteroviral meningitis and infections in infants.

When testing CSF, serum, and throat samples from children with acute illness, PCR demonstrates a sensitivity greater than 90%, while urine testing shows lower sensitivity. In patients with aseptic meningitis, encephalitis, or myelitis, the virus can be detected in stool and respiratory secretions for a longer duration compared to CSF, where it may sometimes be undetectable. EV RNA can be detected in CSF by PCR in most cases of meningitis, but consistently in encephalitis. EV excretion from throat and stool samples is prolonged. During the intervening period, detection is not a confirmation of infection. If detection occurs in stool or throat samples, it should be interpreted carefully because it may not establish causation and could simply reflect extended excretion or carrier status. The use of reverse transcription polymerase chain reaction or nucleic acid sequence-based assays based in clinical samples enables detection of enterovirus genome. Molecular techniques have also been adapted for much wider access in selected clinical specimens. Moreover, the use of genetic material that encodes specific regions of subtype—neutralization epitopes has allowed for serotyping through geographical methods. EV-VPI gene proved serotypes neutralization at a close relationship. Other samples include rectal swab, urine, conjunctival swab, blood, bronchoalveolar lavage, vesicular fluid, nasopharyngeal swab, cerebrospinal fluid, and biopsy.

11.3.2 Sequencing

Traditionally enterovirus (EV) typing was done by sero-neutralization. But now sequencing the VP1 capsid protein gene is the standard method for EV typing. This involves PCR amplification followed by sequencing of the approximately 900 nucleotides of the VP1 gene which encodes a type specific neutralizing epitope. The full VP1 sequence of about 900 nucleotides is required for accurate typing and is essential for new types. Knowing the strain or type of enterovirus is important for epidemiological surveillance, outbreak investigation, and detection of mutations related to resistance or virulence.

11.3.3 Proteomics

Studying proteomics involves discovering and studying proteins to gain insights, into the characteristics of the virus itself. This method often depends on using spectrometry to detect proteins for diagnostic purposes. Techniques such as mass spectrometry, 2 D gel electrophoresis, and phosphoproteomics are utilized to recognize proteins explore interactions between viruses and hosts as well as investigate post translational changes that affect viral reproduction and evasion of the immune system. By analysing the enterovirus proteome potential targets for treatment and indicators, diseases can be pinpointed. Identifying proteins, in samples taken from patients like fluid (CSF) and throat swabs can assist in pinpointing important markers associated with the severity of the virus and could be beneficial, in determining treatment plans and predicting outcomes. Initially proteomics is frequently utilized compared to PCR for diagnostics; however it offers significant insights into the virus actions.

11.3.4 Lateral Flow Methods (LFAs)

Lateral flow assays were designed to detect enterogenic antibodies and antigens because they are rapid, point-of-care immunoassays. Lateral flow assays are simple to perform, portable, and provide results in a short time, usually within 10–20 min. These tests are often used in field settings or for quick initial screening of enterovirus infections, particularly when a fast diagnosis is required. While they are convenient, these tests may have lower sensitivity than PCR or sequencing methods, and there is a possibility of false negatives or positives. Lateral flow assays are molecular techniques that complement each other and can be used in combination because they provide a complete diagnostic approach for enterovirus infections. PCR continues to be the gold standard for detecting enteroviruses, but sequencing and

proteomics techniques provide further insights into the virus's behavior. On the other hand, immunological and lateral flow methods offer fast and practical alternatives for detection.

11.4 Future Prospects and Emerging Approaches

Future advancements in the field suggest that increasing attention on technological improvements and current inefficiencies will enable the deployment of more effective, wider reach, and inexpensive systems. The following areas highlight key developments and their promise to revolutionize the field:

11.4.1 Next-Generation Sequencing and Metagenomics

Next-generation sequencing (NGS) has reorganized the whole structure of molecular biology; enabling the researchers to carry out unlimited analysis of viral genomes in an accurate manner. In case of enteroviruses, NGS contributes the large scope of data, which gives an opportunity to study genetic diversity of many emerging strains, as well as the resistance encoding mutations (Kasibhatla et al. 2016). Metagenomic strategies broaden the scope of NGS applications by providing the capability to detect interspersed levels of all microbes within a sample, which remains helpful in searching for unusual or circulating viruses (Strazzulli et al. 2017). With the ongoing improvements in sequencing capabilities, there is also an effort to create affordable devices that have the potential to make these tools more accessible. Devices such as MinION are useful in everyday genomic analysis; representing a good step towards nucleic acid sequencing (Buermans and den Dunnen 2014), (Patel et al. 2018). Faster and cheaper testing is becoming more needed especially in the populations that are more affected. In the future, these tools could become indispensable for outbreak investigations and large-scale epidemiological studies (Sabat et al. 2013).

11.4.2 Point-of-Care Molecular Diagnostics

More inclined towards rapid testing in underserved regions, the development of point-of-care (POC) molecular diagnostics have been able quite fast and lead to the development of portable devices. Some methods like inexpensive developing loop-mediated isothermal amplification (LAMP) approaches have already shown their ability to detect the RNA of enterovirus with a high degree of sensitivity and

specificity lacking advanced laboratory instrumentation as well (Shang et al. 2023; Moehling et al. 2021). The fact that POC diagnostic devices can be incorporated into portable electronic devices like smartphones or hand-held readers increases their application. For instance, in situations where a smartphone device is used as a platform, results can be interpreted via networks hubs and the data transferred in real time which helps in rapid response measures during outbreaks and improves management of diseases in areas hard to reach.

11.4.3 Artificial Intelligence and Machine Learning

Artificial intelligence (AI) and machine learning (ML) algorithms have great prospects in transforming molecular diagnostic science. They can sift through enormous volumes of data produced by sequencing, proteomic, or epidemiological studies to notice trends, anticipate viral mutations, or help best design diagnostic protocols. For example, it is possible to train ML algorithms to distinguish pathogenic from non-pathogenic strains employing pathogens which decreases false negatives or positives (Ahmed et al. 2020).

Additionally, these AI-supported systems can integrate the diagnostic data with patient's information and provide clinical workers with those insights which are helpful in treatment and lead to individualized programs. There are so vast number of data that can be generated, AI is anticipated to solve the problems to deal with such data through integration and lay decision making for different gaps in delivering healthcare in resource constrained settings.

11.4.4 CRISPR-Based Diagnostics

The adaptation of CRISPR-Cas systems to the diagnostic purposes has added more possibilities for quick and ultra-sensitive methods of viral detection. SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) and DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) are techniques that leverage the specificity of the CRISPR-Cas protein to assist in the detection of viral RNA or DNA at low concentrations (Wang et al. 2023).

There are several prospects of the CRISPR-based diagnostics including certain basic equipment requirement, short turnaround time, and low number of targets at one place at a time. With the advances of technologies, in the future they could be useful PCR alternatives for Point of Care uses, especially in the low resource areas (Del Giovane et al. 2024).

11.4.5 Nanotechnology and Advanced Proteomics

Nanotechnology has the potential to revolutionize molecular diagnostics by improving sensitivity and specificity. For example, nanoparticle-based assays are capable of detecting viral proteins and antigens in very low concentrations which lead to diagnosis at an early stage and a better management of patients' care (Arias-Hidalgo et al. 2022). Mass spectrometry based proteomic approaches can also be used to identify unique biomarkers associated with specific strains of enteroviruses. Such biomarkers could be useful in designing specific tests and treatments that would target the particular clinical manifestations of enteroviral diseases (Shuken 2023).

11.4.6 Multiplex Diagnostic Platforms

In adding to the strength that these multiplex diagnostic platforms exhibit, their development tackles overlapping clinical presentations caused by pathogens distributed around the world. For instance, if there's a case where enteroviral infections have to be treated, clinicians need to differentiate between the etiologies of aseptic meningitis, such as herpesviruses or arboviruses is critical for effective treatment (Saarinen et al. 2020). Multiplex platforms can also support documentation of co-infections, which are gaining further attention because of their contribution to disease severity and treatment regimes. Such a diagnosis can help strengthen clinical agreement towards improving patient management, as well as the final outcomes.

11.5 Global Collaboration and Standardization

Enteroviral diseases can be effectively eradicated by taking the necessary steps at the global level to make sure that diagnosis is performed in the same manner, that relevant information is shared and that new technology is not only available but is also used in practice. Collaborations that have an international nature not only help with conceptualizing the practice but also includes WHO, or regional public health networks in the advancement of war against enteroviruses (Fischer et al. 2022). Trainings on one hand, and physical infrastructure building on the other hand, also ensure that the populations that are remote or have a low socioeconomic status do not miss on the service that has been advanced to them.

11.6 Focus on Vaccines and Therapeutics

Much as diagnostics of enteroviral infections is crucial, the ultimate goal is to reduce the chances of acquiring the infection and also improve the methods currently in place to treat the infection. For example, target regions with many variants could be useful in creating vaccines, and unravelling their usefulness through clinical vaccine trial studies. They also facilitate post-vaccination monitoring to ensure long-term protection (Laajala et al. 2020). Additionally, the combination of diagnostic approaches with therapeutic approaches, which includes the design of antiviral drugs, is also likely to improve the comprehensive capability of enteroviral disease management. The molecular diagnostic of the viruses responsible for the development of enteroviral disease have greatly improved in addressing the weaknesses that many conventional approaches are beset with. Nevertheless, taking full advantage of these developments would still involve a commitment to research, development policies, innovations, and health equity ideals worldwide. The NGS, CRISPR-based diagnostics, and AI technologies are bound to change the entire diagnostic scenario (Chanchal et al. 2024). Apart from these new technologies, efforts towards enhancing access and affordability of resources approaches will guarantee the realization of the goals of molecular diagnostics. Furthermore, such innovations along with a strategic integration of cutting-edge technologies with global health initiatives present great advancement measures to be employed in combating enteroviral diseases. Sustaining a focus on innovation, accessibility, and equity, the scientific community will be able to leverage the advancements in molecular diagnostics to subsequently translate into better patient management and public health outcomes globally.

11.7 Conclusion

One of the most important efforts to combat enteroviral infections is molecular diagnostics. Enteroviral infections are a broad category of RNA viruses that cause a variety of disorders. From the simplest of the upper respiratory symptoms to the most severe neurological disorders, the clinical manifestations of enterovirus underline the urgent need for accurate and timely diagnostics. Traditional methods, such as virus culture and serological tests, while historically significant are now rendered absolute with the rise of new molecular techniques as their sensitivity, specificity, and speed in testing are simply inadequate.

The virus or the immune response of the host are the primary factors which traditional diagnostic approaches consider. Culture of virus for instance may take days to weeks and certain strains or low viral loads may still remain undetectable. This is also the case with serological tests as they depend on antibodies detection which may not exist at the early stages of disease or in the immuno-compromised individuals. These shortcomings cause delays in crucial decision making, and in turn,

the end results are not satisfactory for the patients, in severe or fast progressing conditions.

In contrast, molecular approaches have changed the scope of enteroviral infections diagnosis. Now, techniques such as polymerase chain reaction, reverse transcription PCR, quantitative PCR allow to find viral RNA in specimens with low quantity of the virus, therefore making the process faster and more effective, even for patients with low viral loads. On the other hand, sequencing technologies present the best opportunity to investigate viral diversity in greater depth, through new strain and mutations detection and in virus related epidemiology. Proteomic and immunological assays allow the detection of viral proteins and antigens, thus improving the flexibility of the diagnostic workflows. Moreover, the design of lateral flow assays has evolved challenge point care diagnostics enabling easier detection of enteroviruses in the field especially for resources constrained areas.

However, regardless of the benefits they offer, molecular diagnostic methods face a number of intrinsic disadvantages. Their high expenses, dependence on advanced laboratory facilities, and highly skilled personnel normally restrict their mass application, particularly in low resource settings. Interpretation of the results can be an obstacle, especially in the presence of many infections, low viral loads or residual viral RNA in patients who recovered, as these factors may lead to false positives, reduced assay sensitivity or difficulty distinguishing between active infection and past exposure. Additionally, since enterovirus exhibit a wide genomic variability, improvement in molecular diagnostic techniques are needed regularly to ensure accuracy and reliability of the diagnosis.

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Chapter 12
Advanced Molecular Techniques in Diagnosis of Viral Diseases

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Abstract Nucleic acid-based viral diagnostic is a gold standard approach for the detection of number of viral pathogens offering exceptional sensitivity and specificity. Clinical laboratories use recent technologies such as sequencing and quantitative polymerase chain reaction for fast and precise identification of number of viruses. The rapid advancement in high-throughput technology has transformed nucleic acid based viral diagnostics and frequently utilized in laboratory testing nowadays. Among them, next generation sequencing (NGS) has evolved from a research instrument to a sophisticated diagnostic method for accurate, high-throughput, cost-effective DNA and RNA sequencing. Additionally, clustered regularly interspaced short palindromic repeats (CRISPR) technology has revolutionized diagnostics and employed in viral identification. Digital PCR is now frequently utilized for the absolute quantification of viral nucleic acid in a sample. Advancements in NGS, CRISPR technology, and digital PCR have greatly influenced viral diagnosis nowadays. This chapter explores the significant developments and application of NGS, CRISPR-based diagnostics, and digital PCR in viral diagnosis. Additionally, artificial intelligence (AI) is now integrated with large data set of viral genomic sequences to offer deep insight. Ongoing advancements in AI, demonstrate potential for enhancing the precision and promptness of viral infection identification.

Keywords Viral diagnostic · Next generation sequencing · CRISPR technology · Digital PCR · AI-based diagnostic · Viral surveillance

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12.1 Introduction

Accurate and rapid disease diagnosis is necessary for effective treatment intervention and epidemiological surveillance. Although diagnostics based on nucleic acid detection are among the most sensitive and specific, most of such assays require expensive equipment and skilled staff. A fast and reliable diagnosis of disease is important for successful treatment and avoiding long-term outcomes. Biomarkers based on nucleic acids are crucial for viral diagnosis as they may be amplified from low level of DNA/RNA, allowing for highly specific detection through complementary nucleotide pairing. Nucleic acid-based diagnostics have emerged as the gold standard for a wide range of acute and chronic diseases, particularly those caused by infectious pathogens. During infectious disease outbreaks, like the coronavirus disease 2019 (COVID-19) pandemic, fast and precise nucleic acid-based testing plays a crucial role in disease control (Prabhakar and Lakhanpal 2020; Quer et al. 2022). Nucleic acid-based diagnostics based on quantitative polymerase chain reaction (qPCR) or sequencing have widely accepted and are commonly employed in clinical laboratories. PCR versatility, resilience, and sensitivity have made it the most widely used method for detecting DNA and RNA biomarkers. Next Generation Sequencing (NGS) is a high-throughput approach used for sequencing DNA or RNA from virus-infected samples. This method necessitates advanced laboratory equipment and trained workers. Additionally, such next-generation diagnostics have single-nucleotide specificity, which is critical for detecting mutations that confer resistance to antiviral medicines. However, the high cost of reagents and instruments for this technology is a limitation for laboratories in resource-constrained environments. In addition, CRISPR-based diagnostics now emerge as a diagnostic method in case of many important pathogens including viruses. CRISPR systems are an essential component of a microbial adaptive immune system. They recognize foreign nucleic acids based on their sequence and cleave it using endonuclease activity linked with the CRISPR-associated (Cas) enzyme. Although CRISPR-Cas systems differ across archaea and bacteria, they are linked by their reliance on CRISPR RNA (crRNA), which directs Cas proteins to recognize and cleave nucleic acid targets. The crRNA can be directed to a specific DNA or RNA area of interest by hybridizing to a complementary sequence, which in some systems is limited to the presence of a protospacer adjacent motif (PAM) or protospacer flanking sequence. CRISPR-Cas systems have been used for a variety of applications, including targeted genome, epigenome, transcriptome editing, nucleic acid bioimaging, cellular event recording, and nucleic acid detection. Overall, the rapidly expanding field of CRISPR-based diagnostics builds on the specificity, programmability, and ease of use of CRISPR technology and intends to create nucleic-acid-based point-of-care (POC) diagnostics (Kaminski et al. 2021). Nanostring's nCounter and DNA microarray technology is an amplification-free method for measuring RNA or DNA content by counting fluorescently labelled probes that bind to target molecules and can be used for various applications, including diagnosis of viral infection. Digital PCR (dPCR) has become an invaluable tool in viral diagnosis due to its precision

and sensitivity. It allows for the absolute quantification of viral genomes in a sample without the need for standard curves, making it highly accurate even when viral loads are low. In viral diagnosis, dPCR can be used to detect and quantify specific viral pathogens, monitor viral load in patients, and assess viral mutations.

A new possible strategy to characterize viral infections combines RNA-Seq and NGS data with artificial intelligence (AI) capabilities. Genetic profiles of the virus can be thoroughly evaluated using artificial intelligence technology, making it easier to detect mutations, variations, and even entire viral genomes. AI, particularly machine learning algorithms, can evaluate massive volumes of sequencing data to identify viral sequences, forecast virus behaviour, and diagnose illnesses faster and more precisely than traditional approaches. In addition, AI systems identify significant information from sequencing data, such as specific genetic markers or patterns indicating viral presence. Train machine learning models (e.g., neural networks, random forests) on labelled datasets when viral presence is known. This allows the model to learn the features of viral sequences. In this chapter, we explore the latest advancements in NGS technology and its application in viral diagnostics. We also discuss recent developments in CRISPR-based diagnostic assays, focusing on how different Cas enzymes are utilized for viral detection. We also discuss the dPCR in viral diagnosis. The integration of AI in managing large datasets for efficient, rapid, and accurate viral infection and disease surveillance is also explored. These fields are highly dynamic, offering significant potential for future improvements with the advancements in technologies.

12.2 Next Generation Sequencing and Viral Surveillance

Available sequencing methods are grouped in first, second, and third generation sequencing. With the invention of PCR introduced by Mullis in 1986 (Mullis et al. 1986) and first generation sequencing methods consisting Sanger (chain termination) and Maxam-Gilbert (chemical degradation) sequencing, it was possible to generate sequences ranging 100–1000 bases in a low throughput manner. Using Sanger sequencing, highly specialized sequencing centre published first large high profile genome sequences of *Mycobacterium tuberculosis*, *Plasmodium falciparum,* and *Homo sapiens* (Cole et al. 1998; Lander et al. 2001; Gardner et al. 2002). This technology when applied to the large genome sequencing resulted in high cost and reliance on its quality and specificity. The Maxam–Gilbert method utilizes toxic phosphate isotopes and associated difficulties in analysing sequences longer than 500 bp, curbed its usage in laboratories. The NGS technology enables the efficient sequencing of large genomes with minimal errors, significantly reducing the time required and the effort involved in the process. Figure 12.1 provides a lucid and systematic representation of the workflow, highlighting each major step in sample preparation and library construction to sequencing and data analysis in NGS.

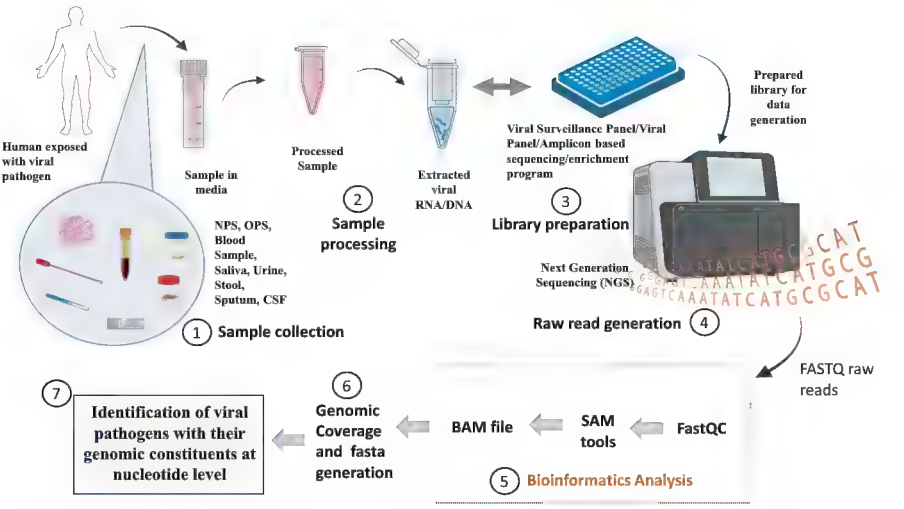
Human exposed with viral pathogen
Sample in media
Processed Sample
Extracted viral RNA/DNA
Viral Surveillance Panel/Viral Panel/Amplicon based sequencing/enrichment program
Prepared library for data generation
NPS, OPS, Blood Sample, Saliva, Urine, Stool, Sputum, CSF
1 Sample collection
2 Sample processing
3 Library preparation
Next Generation Sequencing (NGS)
Raw read generation 4
FASTQ raw reads
5 Bioinformatics Analysis
FastQC
SAM tools
BAM file
6 Genomic Coverage and fasta generation
7 Identification of viral pathogens with their genomic constituents at nucleotide level

Fig. 12.1 Application of NGS in viral diagnostic and analysis of genomic constituents to accurately identify key mutations associated with disease severity. Sample was collected from patient and the RNA or DNA, depending on whether the focus is on RNA viruses (such as influenza or SARS-CoV-2), or DNA viruses were extracted from processed sample. Using NGS technologies high-throughput sequencing raw data were generated. After trimming and processing final data sets were produced. These nucleotide sequences were subsequently aligned with reference genomes or subjected to de novo assembly methods for identifying novel viruses. NPS (Nasopharyngeal Swab), OPS (Oropharyngeal Swab), CSF (Cerebrospinal Fluid)

Next Generation Sequencing (NGS) NGS methods include second generation and third generation sequencing tools and technique (Fig. 12.2). Development of the second-generation sequencing allowed the sequencing with improvements that bear low cost, less time consuming, and accuracy. These methods encompass two major categories: (i) sequencing by hybridization and ligation and (ii) sequencing by synthesis (Slatko et al. 2018). Second generation sequencing has two broad steps involved in all methods: (i) template preparation (Nucleic acid extraction, library preparation, and template amplification) and (ii) sequencing. This highly sensitive technique requires only a minimal amount of viral RNA (<200 ng) to generate a genomic consensus sequence and identify single nucleotide polymorphisms (SNPs) in the analysed samples (Marston et al. 2013).

Sequence by Synthesis (SBS) This method consists of pyrosequencing (Roche 454 method) involves sequencing by reversible termination (Illumina, SOLİD/sequencing by oligonucleotide ligation and detection), and sequencing by detection of hydrogen ions (Ion Torrent sequencing) (Ambardar et al. 2016). These methods have suppressed limitations encountered in former technology and enhanced the capacity to perform sequencing of the entire genome (Heather and Chain 2016). NGS is a massive parallel sequencing technology providing ultra-high throughput and scalability within a limited time, applicable to both RNA and DNA sequencing.

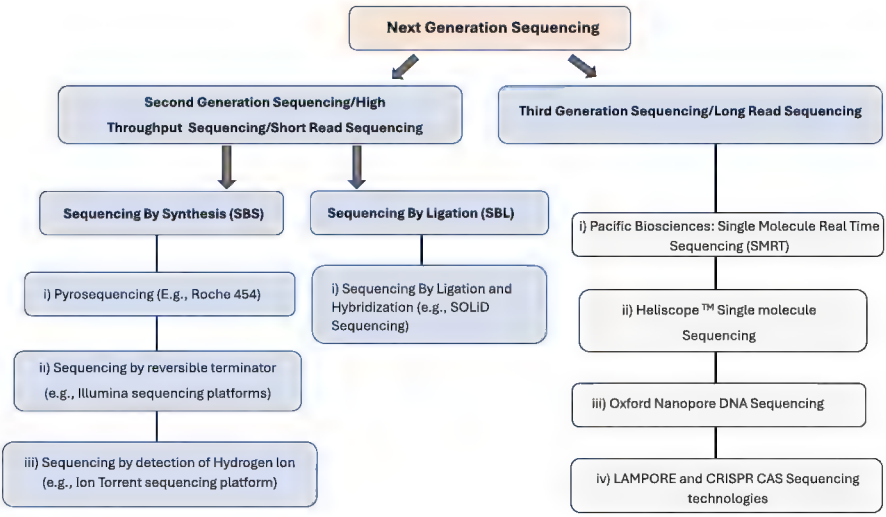
Next Generation Sequencing
Second Generation Sequencing/High Throughput Sequencing/Short Read Sequencing
Third Generation Sequencing/Long Read Sequencing
Sequencing By Synthesis (SBS)
Sequencing By Ligation (SBL)
i) Pyrosequencing (E.g., Roche 454)
ii) Sequencing by reversible terminator (e.g., Illumina sequencing platforms)
iii) Sequencing by detection of Hydrogen Ion (e.g., Ion Torrent sequencing platform)
i) Sequencing By Ligation and Hybridization (e.g., SOLiD Sequencing)
i) Pacific Biosciences: Single Molecule Real Time Sequencing (SMRT)
ii) Heliscope™ Single molecule Sequencing
iii) Oxford Nanopore DNA Sequencing
iv) LAMPORE and CRISPR CAS Sequencing technologies

Fig. 12.2 Next generation sequencing can be divided into second and third generation sequencing based on technologies and principal utilized in the sequencing process

Pyrosequencing Picotiter plate is prepared by entrapping the clonally amplified beads in the wells. DNA polymerization reaction results in release of Pyrophosphate (PPi) is detected and serves as an indicator of base incorporation. Pyrosequencing is a method involving sequential addition and removal of each of the four bases, enabling detection of the bases incorporated into the daughter DNA by DNA polymerase, which triggers enzymatic cascades that generate light signals proportional to the released pyrophosphate.

Sequencing by Reversible Termination Illumina sequencing platforms use a process of clonal amplification, which involves bridge PCR, followed by reversible terminator sequencing (Ambardar et al. 2016). In this process, modified nucleotides with fluorescent labels are incorporated into the DNA strand being synthesized, detected, and then prepared for the next cycle. Each cycle consists of the three steps that are: (i) incorporation of the complementary reversible terminator (R.T.) nucleotide by mutant DNA polymerase onto the flow cell-attached DNA strand, (ii) detection of fluorescence signals for each base, and (iii) restoration of the 3′-OH group by cleaving the terminating moiety and reporter molecule (Berglund et al. 2011). This approach enables accurate sequencing with minimized bias and errors, especially in challenging homopolymer regions Illumina's platforms, including HiSeq and MiSeq, support paired-end sequencing, which sequences DNA from both ends, providing higher coverage and data accuracy. This approach is particularly useful for detecting genomic rearrangements, repetitive sequences, and novel transcripts, making it valuable in applications like RNA-Seq (RNA-Sequencing). Recent advancements include patterned flow cells in systems like HiSeq X Ten, offering exceptional throughput with uniform cluster formation, and the partnership with

10x Genomics to develop technologies like GemCode, enabling long-read sequencing up to 100 kb. This microfluidic system increases read length and sequencing speed, enhancing the capabilities of Illumina platforms for complex sequencing tasks.

Sequencing by Detection of Hydrogen Ions This sequencing method detects hydrogen ions released during nucleotide incorporation, independent of altered bases, enzymes, or optical detection. Template DNA, after library preparation and clonal amplification, binds to Ion Sphere particles in microwells, with each well containing a single particle. A single kind of dNTP is added at a time, and its incorporation triggers an ISFET (ion sensitive field effect transistor) ion sensor, which converts pH changes into digital signals in real time. This method is known as Ion Torrent, a semiconductor sequencing and offers a rapid, cost-efficient, and enzyme-free approach that eliminates the need for modified bases. However, it shares with Roche 454 the challenge of error-prone homopolymer sequencing, though improvements like Hi-Q polymerase have enhanced accuracy and reduced errors. Ion Hi-Q sequencing is particularly useful for whole-genome sequencing and transcriptome sequencing (Ambardar et al. 2016).

12.2.1 Sequencing by Hybridization and Ligation (SBL)

Sequencing by ligation forms the basis of the SOLiD sequencing platform. Unlike sequencing by synthesis (SBS), which uses DNA polymerase, SOLiD relies on DNA ligase to detect base-pair mismatches. Template DNA is fragmented, attached to adapters, and clonally amplified on beads via emulsion PCR/emPCR. These beads are then bound to a glass plate. The sequencing process involves hybridizing and ligating fluorescently labelled oligonucleotide probes to the DNA template. Probes, which contain one or two bases followed by degenerate and universal bases, are ligated to a short primer on the target DNA. Fluorescence imaging detects the ligated probes, and non-ligated probes are washed away. The fluorescent labels are cleaved after detection, allowing for repeated cycles of ligation. Multiple rounds of sequencing with primers of varying lengths reveal the complete DNA sequence. SOLiD read length, initially limited to 35 bp, has improved to 85 bp but still poses challenges in read assembly and sequencing time. Modified platforms for sequencing by hybridization are being developed, to be addressed in third-generation sequencing technologies.

Third Generation Sequencing Methods These methods enabled faster and cheaper sequencing with longer read length. These methods do not require amplification and allow real-time analysis without fragmenting the DNA. Third-generation DNA sequencing methods have Oxford Nanopore Technology and Pacific Bioscience. Second-generation sequencing platforms have two main limitations: short read lengths requiring assembly through bioinformatics tools and PCR bias from clonal

amplification. Third-generation sequencing (TGS), or single molecule real-time (SMRT) sequencing, addresses these issues by sequencing single DNA molecules, minimizing biochemical use and enabling nanoscale processes. TGS platforms include Helicos, Pacific Biosciences, and Oxford Nanopore, with each offering unique sequencing technologies.

Pacific Biosciences Single Molecule Real Time Sequencing In 2010, Pacific Biosciences launched SMRT (Single Molecule Real-Time) sequencing, a nanotechnology-based platform that enables real-time DNA sequencing with exceptionally long read lengths of up to 40 kilobases, although its accuracy trails that of second-generation sequencers. However, it has a high error rate. In this technology, zero-mode waveguides are nanophotonic confinement structures with a circular aperture within an aluminium cladding layer deposited on a silica substrate. At their base, the high-fidelity φ29 DNA polymerase, along with a single-stranded DNA template, is immobilized.

When light passes through a small aperture, there is an exponential decay of the optical field within the chamber. The observation volume inside an illuminated ZMW is approximately 20 zeptoliters (20×10^{-21} L). Within this small volume, the activity of DNA polymerase incorporating nucleotides can be easily detected. The instrument's optical system captures the signals generated during the incorporation of dye-labelled nucleotides by φ29 DNA polymerase during sequencing (Mardis 2013).

Helicos' system simplifies library preparation by avoiding ligation and amplification, directly sequencing DNA and RNA using fluorescently tagged nucleotides. In this technology, DNA is sheared, poly-A tailed, and followed by blocking at 3'OH end with help of thermal transferase and a dideoxynucleotide. A flow-cell containing oligo-dT on its surface is hybridized with poly-A tailed fragments. This process performs the sequencing by synthesis. This technology uses fluorescently tagged nucleotides to sequence DNA fragments that are attached to the flow cell. It allows sequencing as well as quantitation of RNA without converting into cDNA and performs direct hybridization of RNA to the flow cell. This method has limitations, including short read lengths and low data output.

Oxford Nanopore Technology (ONT) uses a nanopore to sequence DNA in real-time, detecting subtle changes in electrical conductivity as the DNA molecule passes through the pore. As the DNA sequence travels through a nanopore with a 1 nm diameter, it changes the electrical conductance of the pore (a protein pore embedded in the polymer membrane. This technology alleviates the need for PCR amplification. Their MinION device, launched in 2014, is portable and field deployable. In 2015, the commercialized MinION variant was optimized and had the capability to mapped 99% of the reference genome and had the sequencing speed of 120–1000 bases/minute. This technology allows to obtain long reads with no amplification, direct detection of target nucleic acid without amplification, and real time access to sequencing information.

LAMPORE Technology LAMPORE Technology integrates loop mediated isothermal amplification (LAMP) with nanopore sequencing, enabling the simultaneous identification of multiple gene regions in a single assay. LAMP is a single-tube DNA amplification method that offers a cost-effective and faster alternative to RT-PCR. Reverse transcription loop mediated isothermal amplification (RT-LAMP) extends this approach to RNA detection through reverse transcription. This method amplifies the targeted region at a constant temperature, achieving enhanced specificity by employing four primers to amplify six distinct sequences within the target gene region. LAMP produces a higher yield of amplified products compared to PCR-based methods, owing to the use of multiple primer sets. LamPORE technology builds on LAMP, integrating sequencing to provide a procedure with high sensitivity and specificity for detailed analysis.

12.2.2 Scope of NGS in Virology

Next Generation sequencing technologies are currently being used for determination of genome diversity, epigenetics, metagenomics, gene expression profiling by RNA sequencing, and protein binding sites. Besides high throughput whole genome sequencing, identification of a new pathogen using metagenomics is also used in microbiology (Jansz and Faulkner 2024). Viruses are contributing to the growing burden of infectious diseases in the human population, including a variety of known viruses with new mutations, as well as rare and previously unrecognized viruses. Some clinical syndromes caused by viruses that cannot be isolated with culture and routine molecular diagnostic methods. Random sequencing and representational difference analysis methods utilized earlier to detect viruses like K.I. polyomavirus, Torque Teno Virus, human G.B. virus, human herpesvirus type-8, bocavirus were time consuming and with poor sensitivity. Implementation of NGS methods with enhanced turnaround time allow detection of viruses without prior knowledge of pathogens. High throughput and fast NGS allow to obtain millions of different DNA sequences, information of genetic alteration and simultaneous assessment of multiple genes (Nafea et al. 2023). Advantages and disadvantages of NGS technologies for the viral diagnosis is summarized in Table 12.1.

Detection of Novel Viruses NGS is advantageous in detecting both known as well as novel viruses with different approaches. Whole genome sequencing (WGS) can be carried out from patient samples directly. This approach not only provides detection benefits but also determines the virulence and presence of drug resistance genes (Nafea et al. 2023).

Detection of Known Viruses Sequencing of specific genes of interest of a known virus instead of the whole genome can be achieved using targeted NGS (tNGS). This method amplifies and enriches the known target before library preparation and sequencing and avoids amplifying sparse microbial sequences in samples rich in

Table 12.1 Advantages and disadvantages of NGS, CRISPR-based diagnostics, digital PCR, and AI-driven technologies for viral diagnosis

Detection methods	Detecting material	Advantages	Disadvantages	References
High throughput sequencing (NGS)	Nucleic acid	Precise and sensitive, not subjected to cross-hybridization, and hence provide high accuracy	Costlier and sequencer machine dependent	Prabhakar and Lakhanpal (2020), Satam et al. (2023)
CRISPR based technology	DNA/RNA	High specificity and sensitivity, rapid and cost effective, multiplexing capability, portable and Point-of-Care use, real-time detection	Off target effect, requiring sophisticated equipment, cost and accessibility, limitations in diagnostic sensitivity	Malekshoar et al. (2023), Zhou et al. (2024)
Digital PCR	Nucleic acid	Absolute quantification without the need for a standard curve, less affected by sample inhibitors and poor amplification efficiency, detecting low-copy-number variants	High costs in terms of specialized instruments and reagents, drop-out in case of larger amplicon	Li et al. (2018), Kojabad et al. (2021), Poggio et al. (2021)
AI-driven technologies	Data sets of genomic content	Enhanced speed, predictive insights, integration with high-throughput technologies, scalability, cost-effectiveness, remote and low-resource applications	Vulnerability to errors, dependence on high level of expertise	Modak et al. (2019), Padhi et al. (2023)

host cell content (Schlaberg et al. 2017), widely used for detection of drug resistance genes (Briese et al. 2015), and detection of mutations associated with pathogenicity (Radford et al. 2012).

Another approach includes metagenomic next generation (mNGS) helpful in identifying all the genetic material present in the samples to detect the presence of potential pathogens. This method with a broad range of detection which allows to identify pathogens, virulence factors, and resistance markers. High cost and long-time are the two drawbacks of mNGS but have shown the high sensitivity.

12.2.3 Viral Phylogenetics and Taxonomy

Viral phylogenetics and taxonomy have evolved significantly due to the rapid accumulation of viral genome sequences. Traditional classification methods based on phenotypic traits are being revised to incorporate genomics data, revealing

unexpected evolutionary relationships among viruses. However, viral phylogenetics faces challenges such as: Rapid mutation rates, horizontal gene transfer, and polyphyly (multiple evolutionary origins) (Kazlauskas et al. 2019; Krupovic et al. 2019). To address these challenges, the international committee on taxonomy of viruses has expanded hierarchical ranks from 5 to 15 and included metagenome-assembled genomes in official classification schemes (Gorbalenya et al. 2020). Advances in metagenomics, high-throughput sequencing, and computational methods are expected to lead to a comprehensive and stable hierarchical taxonomy of viruses, improving our understanding of virus diversity and alignment with host systems.

Genome Epidemiology Viral genome sequencing has revolutionized our ability to monitor viruses that impact ecosystems, and human health. Despite efforts to collect and analyse viral genomes, unknown viruses still pose a significant threat. The COVID-19 pandemic highlighted the importance of preparedness and genomic surveillance. SARS-CoV-2 genome was rapidly sequenced and shared globally, enabling vaccine development and diagnostic assays.

Whole-genome sequencing allowed for tracking of transmission networks, viral evolution, and informed public health responses. The pandemic saw widespread adoption of genomic surveillance, including ONT, offering advantages like minimal infrastructure requirements, real-time data generation, and portability. However, concerns about base-resolution accuracy led to a benchmarking study, which showed ONT's suitability for routine phylogenetic analysis but limitations in detecting rare variants and small structural variations. Improvements in ONT accuracy may address these shortcomings, and future benchmarking will be important for genomic epidemiological efforts. The collective efforts in viral genome sequencing enabled a rapid response to the pandemic and highlighted the importance of preparedness, global collaboration, and accessible technologies for effective pandemic preparedness and response (Ambardar et al. 2016).

Vaccine Development Whole-genome sequencing is now being used to track the genomes of deadly microbes. Advances in NGS, which are both faster and more cost-effective, enable scientists to rapidly generate population-level pathogen data that aids in vaccine development. Unlike the labour-intensive techniques of the 1970s, NGS can sequence hundreds of pathogens in few weeks, uncovering genetic patterns that guide vaccine design. NGS provides detailed information and aiding in development of vaccines by identifying conserved regions of the viral genome. It is used to monitor the genetic stability of desired vaccine strains and the emergence of vaccine escape in advance.

NGS is revolutionizing our understanding of infectious agents' rapid mutation rates, facilitating the development of multivalent vaccines that target diverse viral variants. During the COVID-19 pandemic, NGS played a pivotal role in accelerating vaccine development through rapid genome sequencing. Furthermore, NGS ensures vaccine safety by verifying the genetic stability of modified regions and analysing interactions between vaccines and viruses. Beyond vaccine development, NGS is emerging as a crucial tool in biologics, enabling the detection of

adventitious agents during manufacturing. Traditional viral safety testing methods are time-consuming and rely on animal testing, but NGS offers a faster, more sensitive alternative. Regulatory bodies are increasingly accepting NGS as a replacement for animal-based testing, as evident in the revised ICH Q5A (R2) guidelines. However, broader adoption of NGS for pharmaceutical quality assessment will require additional validation studies and ongoing collaboration between academia and industry (Nellimarla and Kesanakurti 2023).

12.2.4 CRISPR-Cas-Based Sequencing

The emergence of Third-Generation Sequencing technology, combined with CRISPR-Cas-based targeted enrichment methods, has revolutionized genome- wide and targeted sequencing, enabling high-resolution and accurate identification of specific genomic or transcriptomic regions. PacBio technology, known for its long-read sequencing capabilities, can be used for targeted sequencing of genomic regions with high input DNA (e.g., 5000 ng of non-amplified genomic DNA). CRISPR-Cas9 can facilitate targeted enrichment by using guide RNAs to direct Cas9 to specific sites, where it cleaves the DNA, followed by the ligation of sequencing adapters. This method allows for the simultaneous sequencing of multiple targets, reducing the number of sequencings runs and costs. PacBio also supports PCR-based amplicon sequencing, while ONT offers both PCR and PCR-free approaches for targeted sequencing. ONT's CRISPR/Cas9 system is flexible in terms of input DNA requirements, though it lacks multiplexing options. The PCR-free method requires 1000 ng of dsDNA and can detect multiple target regions with high throughput. Targeted sequencing is particularly effective for challenging genomic regions and variants, making it a valuable tool in various research fields, including microbiology, infectious diseases, and biomedical research (Athanasopoulou et al. 2022; Fernandes et al. 2022; Altindiş and Kahraman Kilbaş 2023; Kim et al. 2024).

12.3 CRISPR-Cas Technology in Viral Diagnosis

The CRISPR-Cas technology began with observing the repeating DNA sequences in the genomes of bacteria and archaea. These sequences were crucial in the mechanism of adaptive immunity, which allows the bacteria to remember and target invading viral DNA. This molecular targeting of identifying the specific gene, cutting and editing the DNA, makes CRISPR-Cas an important tool in the development of gene editing (Makarova et al. 2015).

There are few proteins associated with this technique called Cas Enzymes (CRISPR-associated proteins) that can cut DNA or RNA at specific sites. The most used Cas enzymes in diagnostics are Cas9, Cas12, and Cas13. Cas9 is known to be

DNA endonucleases (DNA-cutting enzyme), also referred to as *Spy* Cas9 protein. Cas12 can cut both DNA and RNA, when activated. Single-stranded DNA reporters are the key for generating a signal in diagnostics. Cas13 targets RNA and has collateral cleavage activity, making it useful for detecting RNA viruses. This system targets and cleaves only the ssRNA, not the ssDNA or dsDNA (Liu et al. 2017).

12.3.1 Classification of CRISPR/Cas System

The CRISPR system has been classified into two classes: **(i) Class 1** system requires several effector proteins for the RNA-guided target cleavage. The class 1 system of CRISPR is divided into three types I, III, and IV (Makarova et al. 2015; Koonin et al. 2017). The CRISPR/Cas locus consists of the Cas3 signature gene encoding a large protein with an enzyme called helicase, which is used to unwind DNA-DNA and RNA–DNA duplexes (Brouns et al. 2008). The type III CRISPR/Cas contains the Cas10 signature gene, encoding a multidomain protein with palm domain which targets and cleaves ssDNA (Marraffini and Sontheimer 2008). Type IV system contains CRISPR-associated splicing factor 1 (Csf1), which encodes a ribonucleic protein, but the detailed function of this system is yet to be identified (Koonin and Makarova 2017). **(ii) Class 2 system:** In class 2 system, only one RNA-guided endonuclease is needed to cleave the DNA sequences (Makarova et al. 2015; Koonin et al. 2017). It is divided into types II, V, and VI (Shabalina and Koonin 2008; Koonin and Makarova 2017). The type II locus encodes multidomain protein to target and cleave the dsDNA (Jinek et al. 2012). Type V locus has Cas12 signature gene which is also known as CRISPR extracted from *Prevotella* and *Francisella* 1 (Cpf1), C2c1, or C2c3 protein encoding RuvC (an *E. coli* protein responsible for DNA repair) domain, which cleaves both dsDNA or ssDNA (Zetsche et al. 2015). Type VI contains Cas13 (C2c2) that encodes nucleotide-binding domain of higher eukaryotes and prokaryotes (HEPN), cleaving ssRNA (Abudayyeh et al. 2017). There are various Cas proteins which are associated with the CRISPR mechanism for applications in genetic engineering, biotechnology, agricultural, and medical research namely Cas1, Cas2, Cas9, Cas12, Cas13 etc.

12.3.2 CRISPR as a Diagnostic Tool to Study the Viral Infections

Diagnosis of diseases based on nucleic acid detection techniques for the amplification of viral sequence (DNA/RNA) is considered as a gold standard method being specific and sensitive yet costly. It requires trained personnel, expensive equipment with specific, fluorescent probes, oligo probes, or molecular beacons. CRISPR-based diagnostics technique enables an accurate testing at home as a point of care

test (Kaminski et al. 2021). Many amplification based methods have been developed to cut the cost like **n**icking **e**nzyme **a**mplification **r**eaction (NEAR) (Ménová et al. 2013), recombinase polymerase amplification (RPA) (Piepenburg et al. 2006), nucleic acid sequence-based amplification (NASBA) (Compton 1991), loop-mediated amplification (LAMP) (Notomi et al. 2000). CRISPR/Cas technology can prove to be inexpensive and as sensitive as PCR as they are sequence specific. Class 2 CRISPR systems have been mostly used for diagnostic purposes with the help of several cas enzymes. Class 1 systems can also be used for the diagnostics combining with the components of the class 2 system (such as Csm6 or Cas10) or with the type III complex (Gootenberg et al. 2018; Santiago-Frangos et al. 2021).

In CRISPR/Cas-based diagnostics, Cas12, and Cas13 play a crucial role. Temperature cycling conditions are not required for the collateral cleavage of Cas13 and Cas12 as required in PCR (East-Seletsky et al. 2016; Chen et al. 2018). Also, visual readout-compatible reporters are used to detect collateral cleavage, which replaces the need of thermocyclers and fluorescent reader equipment (Gootenberg et al. 2018). The CRISPR-based detection is adapted to the technologies which are established on detecting viral nucleic acids for DNA and RNA viruses with the help of various sample processing and amplification tools, Cas proteins, and readouts diagnostic methods using CRISPR-Cas.

Assays Based on CRISPR-Cas System (i) CRISPR-Cas9/12/13 systems and (ii) Cas13 (SHERLOCK): SHERLOCK can be used for the detection of RNA viruses like influenza and SARS-CoV-2 as Cas13 is responsible for targeting RNA. The binding of Cas13 with target RNA uses fluorescent or colorimetric reporters and cleaves nearby single-stranded RNA (Kellner et al. 2019). (iii) Cas12 (DETECTR): Cas12 is used to target DNA and produces a fluorescent signal on detecting its target sequence. This method is used for DNA viruses or RNA viruses after cDNA conversion (Tripathi et al. 2022).

The target viral genetic material after extraction is used to amplify via PCR to increase the copy of viral genetic material. The isolated nucleic acid is then mixed with a programmed CRISPR-Cas system to identify specific viral sequences. The CRISPR-Cas complex binds to the target sequence if present (Yuan et al. 2022). A detectable signal in the form of fluorescence or colour change is produced upon binding of CRISPR-Cas to the target nucleic acid. For Cas13 and Cas12, this signal is produced due to the cleavage of reporter molecules (Huang et al. 2022). CRISPR–Cas system is highly sensitive and specific which is designed to target specific viral sequences. Its precision minimizes the false positives and negative results.

Advantages and Future Challenges of CRISPR-Based Technologies The revolution of CRISPR–Cas in a tailor-made format will lead to the discovery of many molecular advance techniques and diagnostic trends. The precision and sensitivity to detect specific viral DNA or RNA sequences makes it an emerging new technique in the field of viral diagnostics. It does not require heavy equipment and its portability makes it a best suited tool for point of care testing (Chen et al. 2023). This technique can be resourcefully used to detect a wide range of viruses by simply

modifying the guide RNA. CRISPR-based tests (SHERLOCK and DETECTR) have been used for the rapid detection of SARS-CoV-2 and can be applied for viral infections like HIV and Hepatitis B/C (Bahrulolum et al. 2023; Hussein et al. 2023). Although CRISPR-Cas systems are designed to target only specific gene sequences yet there is always a risk of editing the unintended regions of gene sequence. This can be avoided by improving advanced computational tools with the precision of guide RNA and accurate Cas proteins (Rabinowitz and Offen 2021). There are some potential risks which are evaluated and need to be addressed to ensure better efficacy and safety. Therefore the policymakers need to establish clear boundaries and standards for the use of CRISPR in the desired area (Shinwari et al. 2018). This can be improved by clinical trials and regular monitoring the effects of CRISPR based techniques (Frati et al. 2024). CRISPR-Cas-based diagnostics hold great promise for rapid, accurate, and adaptable viral detection and a transformed approach for the testing of infectious disease. Advantages and disadvantages of CRISPR–Cas system was mentioned in Table 12.1.

12.4 Digital PCR (dPCR)

dPCR is a technique used for the absolute quantification of nucleic acid targets in a sample and widely employed in research and clinical diagnostic (Yang et al. 2025). Unlike traditional quantitative PCR, digital PCR is a highly precise method that enables accurate quantification of nucleic acid molecules without relying on standard curves. In dPCR, a single PCR reaction is partitioned into thousands of individual micro-reactions, each containing a very small number of nucleic acid molecules, achieved through a limiting dilution approach. The technology utilized poisson statistics, ensuring that the distribution of target molecules across the partitions is random, allowing for precise quantification.

The digital PCR instrument amplifies each of these micro-reactions separately. After amplification, fluorescent probes are used to detect the presence or absence of the target product in each partition. Positive reactions, where nucleic acid templates are present, emit a fluorescent signal (on), while negative reactions, lacking the target nucleic acid, remain dark (off). The term "Digital" is used because the results are quantified digitally, with positive reactions counted as "1" and negative reactions as "0", much like binary code used in digital computers, where logic operations are performed using ones and zeros. Using poisson statistics, the absolute number of nucleic acid molecules present in the sample can be accurately calculated based on the distribution of these ones and zeros across the partitions, providing a highly sensitive and quantitative analysis of nucleic acids in the sample.

Key Innovations in dPCR dPCR has seen remarkable advancements since its inception, shaping the landscape of precise nucleic acid quantification. In 1992, the concept of digital PCR was first conceived and described, laying the groundwork for a revolutionary method of absolute quantification of nucleic acids (Sykes et al.

1992). Researchers began to explore how partitioning a sample into many smaller reactions could enhance PCR precision and sensitivity. In 1999, the first paper using the term digital PCR was published, bringing the concept to the forefront of scientific research (Vogelstein and Kinzler 1999). This marked a crucial milestone in the development of dPCR, sparking interest in its potential for accurate quantification in various fields, including diagnostics and research. In 2006/2007, the first commercial dPCR systems were introduced, utilizing microfluidic chips and microarrays. These systems allowed for the partitioning of PCR reactions into small microenvironments, enabling high-throughput, precise analysis (Griffiths and Tawfik 2006). Microfluidic technology played a key role in reducing the scale of PCR reactions, making them more accessible and efficient. In 2010, a new wave of innovation brought spinning microfluidic discs as the foundation for commercial dPCR systems. These discs allowed for even more precise control over the partitioning process and further refined the accuracy and scalability of dPCR technology. In 2011, another groundbreaking development occurred with the introduction of water-oil emulsion/droplets in commercial dPCR systems. This technique, often referred to as droplet-based digital PCR, provided a highly efficient and robust method for partitioning samples, ensuring that even rare nucleic acid targets could be detected with unparalleled sensitivity. In 2017, a microplate-based dPCR system was introduced, marking a significant leap forward for dPCR in terms of convenience and scalability. This system allowed researchers to run hundreds of reactions simultaneously in a familiar format, making it easier to integrate into high-throughput workflows, while still maintaining the precise quantification capabilities inherent to dPCR. In 2020, the launch of the nanoplate-based dPCR system represented the next step in miniaturization and efficiency. This cutting-edge technology offered an even more compact and advanced platform for digital PCR, further enhancing its applicability in both research and clinical diagnostics. The digital PCR breakthrough is depicted in Fig. 12.3.

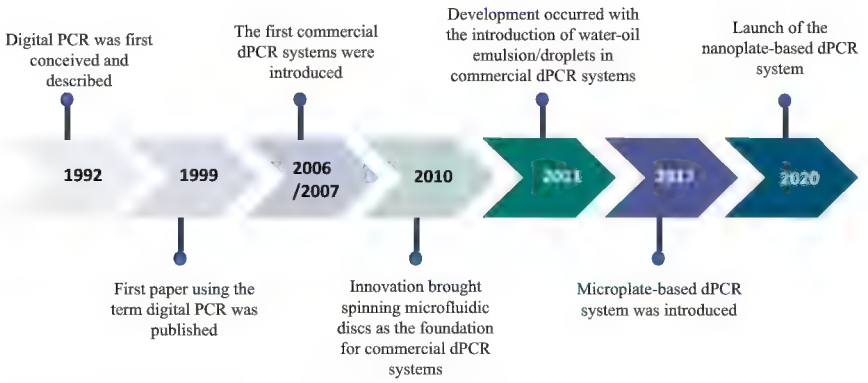
Digital PCR was first conceived and described
1992
1999
First paper using the term digital PCR was published
The first commercial dPCR systems were introduced
2006 /2007
2010
Innovation brought spinning microfluidic discs as the foundation for commercial dPCR systems
Development occurred with the introduction of water-oil emulsion/droplets in commercial dPCR systems
2011
2017
Microplate-based dPCR system was introduced
Launch of the nanoplate-based dPCR system
2020

Fig. 12.3 Milestones in the development of dPCR technology

These milestones in the evolution of digital PCR highlight how the technology has continuously improved, driven by innovations in microfluidics, system design, and partitioning methods. Today, dPCR is a powerful tool used across many fields for its ability to provide absolute quantification of nucleic acids with unprecedented sensitivity and accuracy. The process begins with nucleic acid extraction. PCR primers and probes are then optimized for performance, and fluorescent dyes enable detection during amplification. The sample is prepared similarly to real-time PCR and partitioned into thousands of individual reactions, each containing ideally zero or one template molecule. Partitioning methods include liquid droplets or physical partitions like microwell plates. Successful digital PCR requires random target distribution, uniform partitions, and clear discrimination between positive and negative reactions. For accurate quantification, clinical samples require 10,000 to 100,000 partitions to apply Poisson's law.

12.4.1 Digital PCR Application in Viral Diagnosis

The application of dPCR techniques in diagnostic virology includes several key uses, such as the characterization of reference standards used in qPCR or in sequencing/NGS library preparation (Gleerup et al. 2025). These methods are also crucial for the quantification of viral pathogens, especially when reference standards are not readily available. They offer more accurate quantitation in samples with the presence of inhibitors, like stool samples, which can pose challenges for traditional methods. These techniques are employed in environmental quality monitoring, particularly in surveillance of sewage samples, helping detect potential contamination. Additionally, they are essential for monitoring antiviral therapy, detecting drug resistance, and identifying rare mutations, especially in the background of a wild-type strain (Sancha Dominguez et al. 2024).

12.4.2 Advantages, Limitations, and Challenges of dPCR in Diagnostics

Digital PCR allows for absolute quantification without the need for a standard curve, which increases its precision. The technology also improves interlaboratory comparability, ensuring consistent results across different labs. Additionally, dPCR is less affected by sample inhibitors and poor amplification efficiency, making it more reliable in challenging conditions. It provides more precise results, especially when detecting low-copy-number variants that are difficult to identify with other techniques. Furthermore, digital PCR generates a linear response to the number of copies present, making it highly sensitive to small fold-change differences in the sample. dPCR has a limited dynamic range, which can make it less effective at

detecting high-copy targets. The method also requires careful attention to sample dilution, as improper dilution can impact the accuracy of results. Additionally, the costs associated with digital PCR are significantly higher than traditional PCR, both in terms of the instrument and the reaction costs. The technology also necessitates the use of specialized instruments and reagents, which can further add to its expense and complexity. In clinical microbiology, applications require a high level of accuracy, which can be challenging to achieve with digital PCR. One issue is molecular drop-out, which can lead to reduced accuracy, particularly when working with larger amplicons. The throughput of digital PCR can vary depending on the system, but it is generally lower than that of traditional PCR. There is also a higher risk of contamination due to the more involved handling and sample preparation processes. Furthermore, digital PCR is inherently more complex to perform, often requiring increased hands-on time and the execution of multiple steps to ensure accurate results. There are several other important considerations when using digital PCR. One challenge is the lack of consensus on results across different dPCR platforms, which can lead to inconsistencies in data. Advantages and disadvantages of digital PCR were mentioned in Table 12.1.

12.5 Artificial Intelligence (AI) in Viral Surveillance and Diagnostics

AI has become an important tool in biomedical sciences nowadays. During the COVID-19 pandemic, AI was playing a great role in genome sequencing of SARS-CoV-2, tracking the viral variants and monitoring the COVID-19. AI is being utilized to support various aspects of the COVID-19 crisis, including epidemiology, molecular research, drug development, medical diagnosis, and treatment (Ghose et al. 2020; Severa et al. 2021; Wang et al. 2023; Zeyaullah et al. 2023; Suyash et al. 2024; Khan et al. 2025). AI-driven approaches offering innovative solutions to emerging infectious disease.

In NGS-based viral surveillance, the generation of high amounts of genomic data necessitates advanced tools for transforming raw data into useful knowledge. This demand has led to the development of AI and machine learning (ML) models, which have revolutionized bioinformatics applications, particularly in the field of viral biology.

A study by Lopez-Rincon et al. (2021), which integrate deep learning with explainable AI techniques to uncover representative genomic sequences in SARS-CoV-2. In this study, they identify the 21-bp RNA sequences of SARS-CoV-2 to design a highly specific primer set. For this, a convolutional neural network classifier was trained on 553 sequences from existing repositories, achieving high accuracy in distinguishing genomes of various virus strains within the Coronavirus family. This methodology provides significant advantages over traditional approaches, as it efficiently identifies promising primer sets for viral detection with

reliable results. These capabilities are invaluable for developing rapid and precise diagnostic methods, particularly in the face of potential future pandemics. The ability to quickly adapt and create specific detection systems ensures timely responses to emerging viral threats, underscoring the indispensable role of AI in advancing viral surveillance and diagnostics (Lopez-Rincon et al. 2021). Major viral outbreaks necessitate the prompt elucidation of taxonomic classification and the origin of viral genomic sequences to enable effective strategic planning, containment, and treatment. A study by Randhawa et al. (2020) identifies an intrinsic genomic signature of the SARS-CoV-2 and utilizes a machine learning-based, alignment-free approach for ultra-fast, scalable, and highly accurate classification of complete SARS-CoV-2 genomes. The proposed method integrates supervised machine learning with digital signal processing (MLDSP) for genome analysis, supplemented by a decision tree for machine learning and Spearman's rank correlation coefficient for result validation. These tools were applied to a large dataset of over 5000 unique viral genomic sequences, totalling 61.8 million base pairs, including 29 SARS-CoV-2 sequences. This study suggests that the alignment-free whole-genome machine-learning approach offers a reliable, real-time solution for the taxonomic classification of novel viral and pathogen genomes (Randhawa et al. 2020).

Detecting highly divergent or previously unknown viruses remains a significant challenge despite its clinical importance. In a study by Tampuu et al. 2019, the development of ViraMiner, a deep learning-based method, addresses this challenge by identifying viruses in various human biospecimens. ViraMiner employs two branches of convolutional neural networks (CNNs) designed to detect both sequence patterns and pattern frequencies within raw metagenomic contigs. This methodology represents the first machine learning approach capable of identifying viral sequences among raw metagenomic contigs from diverse human samples. The proposed model is believed to capture different aspects of genome composition and can serve as a recommendation system for further investigation of sequences labelled as "unknown" by conventional alignment-based methods. Investigating these highly divergent viruses can ultimately expand our understanding of the infectious causes of diseases (Tampuu et al. 2019).

Identifying viral sequences from metagenomic data is essential for downstream viral analysis. However, existing reference-based and gene homology-based methods struggle to efficiently identify unknown viruses or short viral sequences. To address this, researcher developed "DeepVirFinder", a reference-free and alignment-free machine learning approach that leverages deep learning techniques to predict viral sequences in metagenomic data. DeepVirFinder was trained on an extensive dataset of viral sequences, and its accuracy was further enhanced by incorporating millions of purified viral sequences from environmental metavirome samples. This expansion of training data significantly improves the method's ability to predict under-represented viruses (Jie Ren et al. 2018).

A study by Lopez-Rincon et al. 2020 proposes an assisted detection test that combines molecular testing with deep learning. This approach utilizes a state-of-the-art deep convolutional neural network capable of automatically generating features from the viral genome sequence. The method classifies various coronaviruses

with an average accuracy of 98.75% in a ten-fold cross-validation and identifies SARS-CoV-2 with an AUC of 98%, a specificity of 0.9939, and a sensitivity of 1.00 in a binary classification. Their code and processed data are made available for diagnostic use (Lopez-Rincon et al. 2020). In a study by Metsky et al. (2020), assay designs were provided for the detection of 67 viral species and subspecies, including SARS-CoV-2, phylogenetically related viruses, and viruses with similar clinical presentations. These designs are the result of algorithms being developed to rapidly create nucleic acid detection assays that are both comprehensive across genomic diversity and predicted to be highly sensitive and specific (Metsky et al. 2020). Synergistic use of AI and machine learning has proven to be a powerful tool in addressing critical challenges in viral diagnostics.

12.6 Challenges and Future Direction

NGS provide extensive insights into viral genomes and key mutations, allowing for the identification of novel and rare viruses. However, NGS-based diagnostics demand a significant amount of data storage and processing capabilities. Combining AI and NGS enables rapid analysis of large datasets for faster diagnosis, thereby improving diagnostic precision, efficiency, and adaptability. Once trained, AI models may adapt to a wide range of illness, improving the response to viral outbreak. However, the high costs of reagents, equipment, and professional staff may limit access, especially in resource-limited setting. Affordable reagents, portable high-throughput instruments, and AI-powered automation can all assist to improve accessibility and efficiency. Future viral diagnostic research will focus on developing pan-pathogen testing for both known and undiscovered viruses. Integrating NGS with CRISPR-Cas and AI could streamline mass testing during any outbreaks, enhancing sensitivity, specificity, and speed. Global public health can be improved by utilizing diagnostic data obtained by NGS in immunization programs, antiviral therapy, and outbreak management.

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Chapter 13
Molecular Diagnosis Beyond Nucleic Acid Tests (NAT)

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Abstract The current era demands the development of well-designed point-of-care (POC) diagnostic systems capable of rapidly detecting multiple diseases. Nucleic Acid Test (NAT)-based detection has been extensively studied and optimized for high sensitivity and specificity. However, challenges remain in terms of operational ease, high costs, time constraints, and the requirement for skilled personnel. To address these limitations, there is an urgent need to integrate high-throughput techniques such as flow cytometry, mass spectrometry, and nuclear magnetic resonance (NMR) with lateral flow-based or biosensor-based platforms to develop simple, cost-effective, and rapid diagnostic systems for detecting multiple biomarkers. Flow cytometry employs lasers to identify the characteristics of cells, while MS ionizes and NMR magnetize the targeted biomolecules to identify specific characteristics and recognizes structures and interactions of different molecules. A critical step forward involves evaluating and integrating these methodologies, identifying technical gaps, and redesigning these systems into miniaturized, user-friendly versions suitable for POC applications. This chapter provides an in-depth analysis of these methodologies, highlighting key challenges and potential solutions for the advancement of next-generation diagnostic platforms. Research in this direction will enhance our understanding of these technologies and drive innovation toward more efficient and accessible diagnostic solutions.

Keywords Diagnosis · Flow cytometry · Mass spectrometry · Nuclear magnetic resonance · CRISPR-Cas

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13.1 Personalized Lateral Flow-Based Approaches

Lateral flow-based approaches (LFBA) are membrane-based immunoassays that detect target antigens of infectious agents. LFBA is more rapid, point-of-care (POC), specific, and cost-effective. LFBA mainly relies on antibody or antigen-based detection. The viral surface antigens are mostly the antigen biomarkers, and the host humoral immune responses against these viral antigens are antibody biomarkers for detection. The antigen-based approaches are precise to specific virus detection. The antigen present in the virus surface region, either embedded in the envelope or capsid (non-enveloped viruses), provides appropriate confirmation for the presence of the virus. However, the antibody target detection may produce false positive results due to non-specific binding (Vanroye et al. 2021). However, antibody detection aids in understanding patients' history of viral infection. Antibodies produced through the humoral immune response remain in the bloodstream for a few weeks/months.

LFBA are classified under Rapid Diagnostic Tests (RDTs), which impart several advantages over molecular methods. LFBA mainly targets proteins for detection. Target protein over nucleic acid aids in bypassing the amplification step. This provides rapid results without the processing of the samples. It also makes the technique feasible, POC and cost-effective. The specificity stands out equally with the molecular methods (Berger et al. 2021). It lacks sensitivity when compared with molecular methods (Tsai et al. 2018; Berger et al. 2021). However, with advancements like ultra-sensitive RDTs, sensitivity problems may resolve to a greater extent. The ultra-sensitive RDTs consist of highly luminescence fluorescent molecules.

13.1.1 Rapid Diagnostic Test (RDT) Assembly

The chromatography system facilitates the uniform capillary action of molecules extracted from the sample according to their size and shape, and it subsequently interacts with the immune molecules of the biorecognition components (Mattar et al. 2020; Panferov et al. 2023). The assembly comprises an absorbent pad in a reverse flow-through arrangement, a nitrocellulose membrane, a conjugate pad, and a sample pad (Fig. 13.1). The specimen is placed onto the sample pad; all sample waste is absorbed by the absorbent pad, which also supplies unidirectional flow to the technique; the conjugate pad has a tag-labelled antibody against the biorecognition element; and the nitrocellulose membrane has immovable antibodies on the test and control line. The entire assembly is placed inside the cassette, which has multiple zones to observe the buffer, sample, and results.

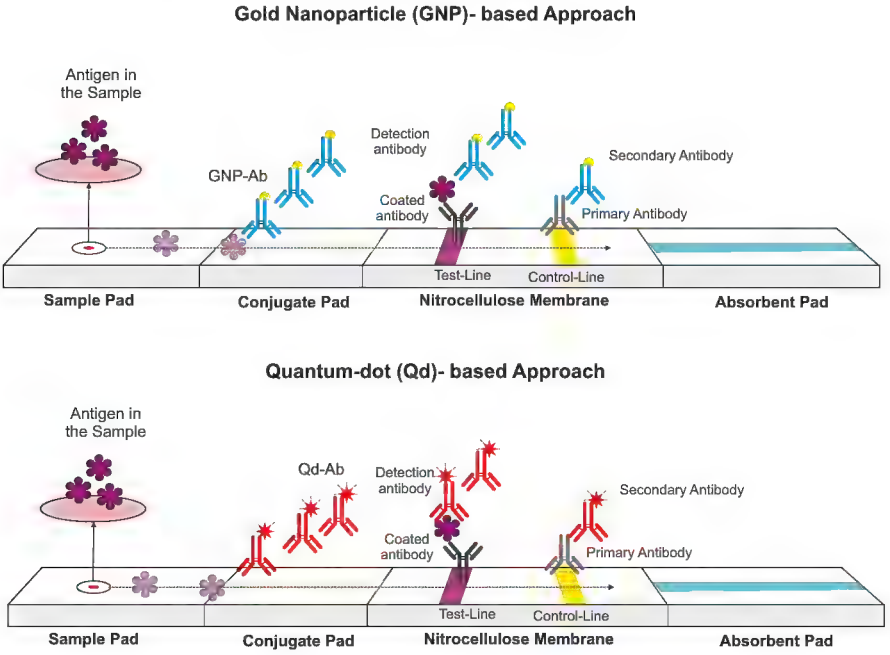

Fig. 13.1 The virtual representation of the assembly of membranes for lateral flow-based assay consists of a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The conjugate pad contains either gold nanoparticles and antibody conjugation or fluorescent molecule and antibody conjugation. Nitrocellulose membrane contains a coating antibody on the test line and an anti-goat antibody produced in rabbits on the control line. The absorbent pad is used for the unidirectional flow of the solution

13.1.2 Role of Different Membranes

The LFBAs are designed with several membranes, i.e. sample pads, conjugate pads, nitrocellulose membranes, and absorbent pads. The sample pad is the first membrane aligned with the flow of the buffer. The sample pad filters out larger particles or impurities from the sample and regulates macromolecule flow through the membrane. This aids in a smooth transition of samples from conjugate pad to nitrocellulose membrane. The sample pad materials are designed and selected in such a way that they can hold the sample and release it, which allows controlled movement of the samples. These are made of glass or cellulose fibre materials (Alam et al. 2021). The conjugate pad holds the detector alloy with the antibodies or antigens in dried form. When the buffer and sample component pass through the conjugate pad, it is rehydrated and interacts with the target analyte from the sample. This conjugation binding at the early stage proceeds in the commencement of the detection. So, at the later stage, it is left to bind to the test line analyte. The conjugate pad is usually made of glass fibres or polyester, which can hold the analyte, have good wicking properties, and release the analyte at a controlled rate (Mirica et al. 2022). The

material design and advancement in the conjugate pad can significantly impact the Lateral flow assay (LFA) performance.

The nitrocellulose membrane is the core platform of the LFA. The test and control line analytes are coated as immobilized elements. The conjugation of the detector with antibodies and target analytes flows in the unidirectional side towards the nitrocellulose membrane. During this movement, it encounters these immobilized antibodies and interacts with them to produce a band, i.e. visualized by the detector alloy. The combination of coated antibodies—target analyte—detection antibodies—detector molecule gets concentrated to visualize the band and suggest a positive result for the target analyte. If the target analyte is absent, this combination will not be formed, and all the components will move unidirectional towards the absorbent pad. This will not produce any band and suggest negative results. A control line combines coating antibody binding with detecting antibodies to produce a band. If these bands are not visible, it is considered an invalid result, suggesting the antibodies or the detector molecule degraded mainly during storage conditions. Nitrocellulose membranes are formed by nitration of the cellulose membrane. The membrane has a specific pore size for effective transfer or immobilization of biomolecules. The biomolecules bind with the membrane through hydrophobic interaction or hydrogen bindings. The membranes become semi-transparent when wet, which helps in better visualization of the detector alloy. The other membranes used in place of nitrocellulose membranes are polyester and polyvinylidene difluoride membranes (Harpaz et al. 2020). However, the nitrocellulose membranes are efficient in LFBA due to its chemical functional group that covalently immobilizes the enzymes, proteins, and nucleic acid and prevents non-specific binding of biomolecules (Harpaz et al. 2020; Alahmad et al. 2021). An absorbent pad is attached at the end of the lateral flow assembly, which directs unidirectional flow to the buffers and sample content. It absorbs the solution from other membranes in around 20–30 min, providing a clear background on the test strip. This helps in better visualization and precise interpretation of results.

The empirical aspect of the LFBA is the binding between the antigen and antibody and the stability of the detector molecule attached to the detection antibody. The critical molecules in the LFBA are coating antibodies, detecting antibodies conjugated with detector molecules, and targeting antigens in the positive samples. The target antigen is the biomarker found for the viral infection. Coating and detection antibodies are produced against the biomarkers. The detector molecule is gold nanoparticles (GNPs). In the case of fluorescent LFBA, the detector molecules are quantum dot (Qd), upconversion nanoparticles (UCNP), and time-resolved fluorescence (TRF) (Díaz-González et al. 2020; Lu et al. 2021; Martiskainen et al. 2021). The detection antibody is conjugated with GNPs and overlay on a conjugate pad. When the positive sample is added to the sample pad, followed by the buffer, the target antigen in the sample binds to the detection antibody conjugation and flows forward. After this successful interaction, the whole combination moves towards the test line on the nitrocellulose membrane. This target antigen of the entire combination binds to the coating antibody immobilized on the test line. As the coating

antibody is immobilized, the complete combination gets hold of that line and produces a band due to the aggregation of GNP molecules in one place. The slower the molecule moves, the better the interaction among each other.

13.1.3 Elements Responsible for Advancement in the Methodology

The LFBA are modified and developed in several ways to establish it as sensitive as molecular methods. Many experimental studies have been performed to increase the sensitivity of LFBA. To work on the sensitivity part of the technique, one must thoroughly comprehend it and be able to identify the various improvised aspects of the process. Some contributing factors are advanced membrane quality, a stable, humidity-controlled environment, buffer chemistry, and the effectiveness of the binding between the antigen-antibody complexes. Slow flow rates can compensate for the loss of some antigens that test line antibodies which cannot connect to by lengthening the time it takes for analytes to bind to one another. Higher antibody concentrations, however, can partially meet the requirements. Up to a point, all of these variables will significantly increase the technique's sensitivity, but it won't reach the molecular methods' standard. To achieve a very high level of sensitivity, the signal/detector molecule is the most vital component that can potentially be a crucial element in LFIA. The ability of the detector molecule to produce visibility can be a vital component in boosting sensitivity, even in cases where the antigen concentration in the sample is low. The GNP chromogenic molecules are made in nanoshells to increase their visibility in low numbers. A strong signal is produced in nanoshell forms mainly due to the combination of several GNPs in a spherical arrangement encircled by a thin metallic shell (Loo et al. 2004). These nanoshell GNP molecules binding to the detection antibody have provided more effective results than conventional LFBA (Zhan et al. 2020). In addition to chromogenic molecules, fluorescence molecules are important in producing high-intensity signals. Compared to chromogenic compounds, the light intensity of fluorescent molecules is higher. The use of fluorescence as a detector molecule like Qd, UCNP, and TRF has increased the sensitivity by ten-fold or more than the existing RDTs (Liang et al. 2020; Acquah et al. 2021; Wu et al. 2021). This molecule can also exist in the form of microspheres or nanoshells, which, when excited increase the photon signals and allow vision at lesser biomarker molecules. In lower antigen concentrations, this will eventually trigger the fluorophore molecule to glow. These nanoshell semiconductor compounds are brighter and produce numerous fluorescence signals due to their narrow emission bands, strong photostability, and high molar extinction. The method will be gradually guided to increase the sensitivity by multiple yields. This leads to the development of ultra-sensitive RDTs. The sensitivity of ultra-sensitive RDTs is very close to that of molecular methods.

13.1.4 Vertical Flow Assay

The vertical flow assay is a new method developed through modification in the assembly of LFA (Kim et al. 2021). The assembly is in a vertical flow, allowing the solution to flow with the aid of gravity. The sample is directly placed on the porous membrane where the antibody is immobilized. The absorbent pad surrounding the membrane absorbs the complete blood sample on the outside, making the porous membrane clear and visible. After that, a buffer is added containing conjugate antibodies attached to GNPs. The absorbent pad slowly absorbs excessive content. This process allows the interaction between the antigen and antibody to take place appropriately, providing a clear, visible result in the form of a dot in positive samples. This new method pin-points better multiplexing than the traditional lateral flow assay (Lu et al. 2023). This method may mark the future of RDTs in the market.

13.1.5 Multiplexing

Multiplexing has advanced to include numerous analytes that can be detected simultaneously on a single strip. This can be accomplished by labelling distinct analytes with different colours or placing multiple test lines—each unique to a different analyte—on the same membrane. This led to the detection of multiple diseases with the same sample. Various designs have been adopted to create division from one sample pad to 3–5 nitrocellulose membrane for high-throughput analysis. The disease subspecies or other important parameters are also detected in this way. Multiplexing LFBA is the future of RDTs with an ultra-sensitive approach.

13.1.6 Smartphone Reader

An effort has been made to incorporate LFAs with digital and smartphone-based technologies. Digital reader devices can quantify and interpret the intensity of test lines more accurately than visual inspection (Liu et al. 2020). Smartphone applications use smartphones' cameras and computing capacity. These applications allow remote diagnostics through image capture, analysis, and interpretation of LFA results.

13.2 Addressing Methodological Bottlenecks for Translational Impact of RDTs

Rapid Diagnostic Tests (RDTs) have many advantages but face challenges that may affect their efficacy and dependability. RDTs may have variable sensitivity and specificity that lead to false positive and false negative tests, respectively. It is challenging to ensure RDT's consistent performance and quality between batches and manufacturers (Bosco et al. 2020). Production variations can potentially impact test results' accuracy and dependability. User errors, such as inappropriate sample collection, faulty test administration, and incorrect result interpretation, may compromise the accuracy of RDT results. Sufficient training of healthcare personnel and consumers is necessary to guarantee proper testing protocols and precise result interpretation. Variability in test results may be caused by inconsistent training. RDTs are susceptible to changes in light, humidity, and temperature in the immediate vicinity. The test components' performance may be impacted by improper handling and storage. Sustaining ideal storage conditions and ensuring tests are administered correctly can be difficult in settings with limited resources. Several RDTs offer qualitative data in place of quantitative data. This constraint may impact the monitoring of disease development or therapy response. RDTs may not be as sensitive in identifying low analyte levels (Tsai et al. 2018; Martiáñez-Vendrell et al. 2022). Improving the technical performance of RDTs, strengthening training and education, ensuring that regulatory and quality control procedures are strong, and encouraging cooperation between manufacturers, healthcare professionals, and policymakers are just a few strategies to address these issues. By tackling these issues, the potential of RDTs to improve global health outcomes can be fully realized.

The LFBA are affordable, specific, sensitive, user-friendly, rapid and robust, equipment-free and delivered to end-users (ASSURED) as recommended by the WHO guidelines for diagnostic kits. RDTs are favoured for their ease of use, rapid, and capacity to deliver results that can be put to use without the need for sophisticated lab equipment or highly skilled workers. They play a critical role in resource-constrained environments, isolated locations, and emergencies where prompt identification is necessary for efficient disease treatment and containment. RDTs play a pivotal role in disease management strategies by enabling timely diagnosis, tracking the efficacy of treatments and direct patient management routes. When it comes to illnesses like tuberculosis, HIV/AIDS, malaria, and respiratory infections, they are very crucial. Veterinary medicine, drug testing, food safety, infectious diseases, STDs, and environmental monitoring are just a few fields that use RDTs. They are multipurpose devices. Their adaptability makes them valuable in diverse healthcare and non-healthcare applications. Despite their advantages, RDTs have limitations, including inconsistent sensitivity and specificity, the possibility of false positives or negative results, and limited quantitative data. Because of these restrictions, results must be carefully interpreted, and confirmatory testing is required.

RDTs provide decentralized testing, which eases the load on centralized laboratories and makes diagnostic services more widely available and effectively run. RDTs facilitate healthcare professionals to diagnose patients more quickly and accurately, leading to improved patient outcomes. Rapid diagnostic tests are essential in contemporary medicine, providing advantages that enhance the efficacy, accessibility, and efficiency of disease diagnosis and treatment. Their importance is demonstrated by their capacity to offer quick, dependable, and affordable diagnostic solutions—especially in situations where standard laboratory services are impractical. RDTs will remain essential tools for enhancing global health and halting the spread of infectious diseases as technology develops.

13.3 Flow Cytometry and Virometry as Emerging Molecular Diagnostic Approaches

In biological and medical research, flow cytometry is a versatile and efficient tool that provides a comprehensive view of the properties and activities of specific cells. Its rapid and precise evaluation of an enormous number of cells makes it an invaluable tool in many domains, such as microbiology, cancer, and immunology. Flow cytometry extensively identifies and isolates individual cells or particles from a mixed population (McKinnon 2018). It suspends cells or particles in a liquid solution and passes them through a laser beam. As each particle traverses the laser beam, it scatters light in specific ways that provide insights into its characteristics. Forward scatter (FSC) measures the light scattered in the forward direction, indicating the particle's relative size. In contrast, side scatter (SSC) measures light at a 90° angle, revealing the internal complexity or granularity of the particle. Additionally, flow cytometry utilizes fluorescence parameters where particles labelled with fluorescent markers emit light when excited by lasers, offering information on specific molecular markers or compositions. This led to diagnosing multiple parameters in one disease or identifying various infections from one sample.

13.3.1 Flow Virometry

In clinical diagnostics, flow cytometry plays a crucial role in identifying and quantifying various cell types, aiding in the diagnosis of multiple diseases. However, traditional flow cytometry encounters challenges when detecting viruses or viral particles due to their small size and the absence of specific surface markers for easy fluorescent labelling. To overcome these limitations, a specialized version of flow cytometry known as "flow virometry" has been developed (Arakelyan et al. 2013). Flow virometry is tailored to detect, characterize, and analyse viruses or viral particles in biological samples. This technique enhances the sensitivity and specificity

required for virus detection, thereby expanding the application of flow cytometry into virology and infectious diseases research (Bonar and Tilton 2017). The ability of flow virometry to handle the unique properties of viruses makes it a valuable tool in understanding areas of virological study. The first successful detection of a virus using flow cytometry was accomplished by Hercher et al. in 1979, where they detected T2 phages (approximately 60 nm in width and 120 nm in length). In these initial studies, the virus particles were fixed using either glutaraldehyde or formaldehyde. Following this, poxvirus and fixed T4 phages were also detected using flow cytometry (Hercher et al. 1979). However, there was still a challenge in detecting smaller viruses. The improvisation in existing technology will be critical to observe for the smallest viral detection. In recent years, the detection of viruses by flow virometry has become more common.

Viruses are minute pathogens that exclusively replicate within host cells. Upon entering a host cell, they hijack the cellular machinery to reproduce and generate proteins, often leading to severe infections and potentially fatal outcomes. Viral infections pose a significant challenge in detection when their symptoms mimic other diseases, complicating accurate diagnosis and delayed treatment. Moreover, viruses exhibit a wide diversity in their structures and genetic makeup. Their high mutation rates, particularly among RNA viruses, facilitate the emergence of new strains that may evade existing detection and treatment methods. Current virus detection techniques include PCR, western blotting, ELISA, and mass spectrometry. However, these methods are not optimized for characterizing viruses at the level of individual particles and cannot assess variability between particles. Electron microscopy, while providing detailed structural information at the single-particle level, is hindered by its time-consuming nature and limited throughput. These traditional approaches also struggle to effectively analyse the heterogeneity of viral subpopulations, differentiate features within these sub-populations, or comprehensively examine the antigenic composition across large viral populations simultaneously.

In response to these challenges, there is a growing need for advanced technologies, such as flow cytometry, specifically designed to detect, characterize, and analyse viruses or viral particles in complex biological samples. Flow cytometry is promising for enhancing our understanding of viral diversity, evolution, and pathogenic mechanisms, advancing diagnostics, vaccine development, and therapeutic strategies in virology and infectious diseases.

The initial step in performing flow cytometry involves virus production, which consists of several methods. The cells are typically transfected with a plasmid carrying the genetic code necessary for virus production. Alternatively, virus particles can be harvested from the supernatant of cultures containing infected cells or specific infected cell clones. Another approach involves infecting cell cultures with the virus and allowing it to replicate within the cells. The second step is virus labelling, which is essential for subsequent analysis. This can be achieved by fusing fluorescent proteins, such as GFP, with viral proteins. This method ensures that the virus emits fluorescence, making it detectable and distinguishable from other particles in the sample. Viruses can also be labelled using antibodies conjugated with fluorophores that target specific viral proteins or host cell-derived surface proteins on the

viral envelope. This dual approach provides flexibility in identifying and characterizing different types of viruses. The final step in flow cytometry is the analysis, where the labelled viruses are examined to gain insights into their characteristics and behaviours. This analysis yields valuable information about viruses' composition, concentration, and behaviour within the sample, facilitating a deeper understanding of viral biology and pathogenicity.

Innovations in fluorescent dye chemistry, including quantum dots, have enabled brighter and more stable labelling of viral particles without compromising their integrity. Sample preparation techniques have also been refined, ensuring consistent fixation, labelling, and preservation of viral integrity. These improvements allow for more reliable results and minimize sample loss and artefacts.

13.3.2 Detectors and Optics

Flow cytometry has greatly enhanced the viral diagnostics and research field through a series of technical advancements, novel applications, and improved analytical capabilities. Modern flow cytometers feature with highly sensitive detectors and advanced optics capable of accurately detecting viral particles as small as 20 nm. Photomultiplier Tubes (PMTs) were traditionally used as detectors; however, recently, more advanced alternatives are being used, such as Photodiodes (PD), Avalanche Photodiodes (APD), Charge-Coupled Devices (CCDs), and Complementary Metal-Oxide-Semiconductor (CMOS) sensors. PMTs are extremely sensitive detectors that transform weak light signals produced by cells into electrical signals by amplifying and signalling them (Snow 2004). Multiple fluorochromes can be detected simultaneously through PMTs' broader wavelength detection capacity. APDs are semiconductor devices with exceptional sensitivity for converting light into electrical signals. Compared to ordinary photodiodes, they have higher sensitivity and work well to detect low-intensity light. Applications demanding great sensitivity and quick response times frequently use APDs. Light is detected by photodiodes, which transform it into an electrical current. Their fast response times and excellent light intensity tolerance are ideal for detecting side scatter (SSC) and forward scatter (FSC) signals. In imaging flow cytometry, CCDs and CMOS sensors take fine-grained pictures of cells. They offer high-resolution images and can identify many fluorescence signals simultaneously (Goddard et al. 2006; Grégori et al. 2012).

13.3.3 Advanced Computational Tools

Enhanced analytical capabilities have further revolutionized the field. Advanced computational tools and algorithms, including machine learning and artificial intelligence, are now employed to interpret complex data sets, identify patterns, and

accurately predict outcomes. These tools also significantly reduce the time required for flow cytometry analysis (Aghaeepour et al. 2013). Various freely available software tools have recently been developed, which further help in rapid and accurate analysis. Moreover, integration with other cutting-edge technologies, such as next-generation sequencing and mass spectrometry, allows for a more comprehensive analysis of viral particles by combining phenotypic data with genotypic and proteomic information.

13.3.4 Technical Constraints and Clinical Implications

While flow cytometry represents a significant advancement in viral diagnostics, it faces several challenges. Technically, achieving the necessary sensitivity and resolution to detect the minute size of viral particles is difficult, requiring specialized equipment and precise protocols. Another complexity involves the calibration and standardization of flow cytometers across different laboratories and the challenge of efficiently labelling viral particles with fluorescent dyes without compromising their integrity. Viruses are biologically heterogeneous, with considerable size, shape, and genetic material diversity. This variability makes detection and analysis more difficult. High mutation rates, especially in RNA viruses, create new strains that may evade established markers. Moreover, the interaction of viruses with host cell components adds another layer of complexity, making it challenging to distinguish free viral particles from those associated with host cells. Analytically, the vast amount of data generated by flow cytometry necessitates advanced tools for accurate interpretation. Developing algorithms to handle the complexity and variability of viral data is essential, especially in standardization protocols, to ensure consistent results. Differentiating viral particles from similar extracellular vesicles or particles present in biological samples requires high specificity and resolution, posing another challenge. The high cost of advanced flow cytometers and necessary reagents is practically limited to accessibility, especially in resource-limited settings. The complexity of the technique also demands specialized training and expert workforce, ensuring that personnel can operate the equipment and interpret data appropriately. Despite these challenges, flow cytometry's potential for advancing viral diagnostics and research is immense. Addressing these technical, biological, and analytical issues through ongoing research and innovation will enhance the accuracy, reproducibility, and accessibility of this powerful technique, making it an indispensable tool in the fight against viral diseases.

The novel applications of flow cytometry are equally impressive. High-throughput screening capabilities have been adapted for large-scale epidemiological studies, vaccine development, and antiviral drug screening. The technique's ability to analyse individual viral particles facilitates the study of viral population heterogeneity, aiding in identifying different sub-populations, understanding viral evolution, and detecting clinically significant minor variants. Moreover, multiparametric analysis now enables the simultaneous measurement of multiple parameters,

providing a comprehensive profile of each viral particle and allowing for detailed characterization and differentiation.

Looking to the future, the incorporation of nanotechnology and microfluidics promises to further enhance sensitivity and resolution in flow cytometry. Recently, significant advancements have been made by combining mass spectrometry and flow cytometry, called mass cytometry, providing high dimensional single-cell analysis (Spitzer and Nolan 2016). The development of portable, user-friendly devices could bring point-of-care diagnostics to remote and resource-limited settings, providing rapid and accurate viral diagnostics at the bedside or in the field. Real-time monitoring systems, capable of continuously observing viral particles in a sample, offer significant potential for studying viral dynamics, monitoring the efficacy of antiviral treatments, and conducting real-time epidemiological surveillance. As technology evolves, flow cytometry is poised to play an even more significant role in the fight against viral diseases.

13.4 Proteomics and MS as Yet-to-Emerge Tools in Molecular Diagnosis

Proteomics, which includes the study of proteins, enables detailed analysis of protein structure, expression, and function. Proteomics technologies have been extensively applied in the fields of diagnosis and treatment. Various techniques like 2-D gel electrophoresis, western blotting, NMR, X-ray crystallography, mass spectrometry, and protein microarray are used to study proteomics. In diagnosing multiple diseases, immunoassays are commonly used, but they often face limitations in sensitivity and cross-reactivity. Therefore, there is a need for alternative techniques to improve the accuracy and effectiveness of diagnosis. Mass spectrometry (MS) has emerged as a crucial diagnostic tool due to its high sensitivity, specificity, and ability to detect biomolecules accurately. MS is an analytical technique used to identify and quantify molecules based on their mass-to-charge ratio of ions (Urban 2016). The integration of MS with other proteomics techniques further enhanced its diagnostic potential.

13.4.1 Mass Spectrometry

The basic principle of MS is based on measuring the mass-to-charge ratio (m/z) of ions to identify and quantify chemical substances. The sample preparation is a crucial step in MS to ensure reproducible and high-quality results. Proper handling and processing of samples are essential to avoid variation in results. Different steps are required for various samples, depending on their nature (Peukert et al. 2012). The process involves dilutions in solvent and extraction and purification of desired

analytes from the sample. The protein in the samples can be broken down into smaller pieces using the enzyme trypsin, which cleaves at specific amino acid sequences to form peptide fragments (Gillet et al. 2016). The extracted fragments can be derivatize with specific chemical compounds to increase the volatile nature of the analyte, especially in the case of Gas Chromatography MS (GC-MS). The chemical compounds can be trimethylsilyl derivatives for alcohol and phenol, methyl iodide, benzyl bromide for amines, sulfuric acid for esterification, etc. The fragments are then ionized to form charged particles (ions). This can be done using various methods, such as Electron Ionization (EI), Electrospray Ionization (ESI), and Matrix-Assisted Laser Desorption/Ionization (MALDI). EI is a process where electrons are bombarded on the sample to create ionization among the fragments (Maciel et al. 2022). ESI is a process where solvents like acetonitrile, methanol, water, acetic acid, or ammonium acetate are used for the ionization of the fragments (Eberlin et al. 2011). MALDI used lasers in a given matrix to create ionization. Once ionized, the ions are accelerated to have the same kinetic energy and are deflected by a magnetic field based on their mass and charge ratio (Croxatto et al. 2012). The extent of deflection is inversely proportional to the mass of the ion; hence, the ions with lesser mass deflect more, while heavier ions deflect less. The filters or traps used in these conditions are a quadrupole mass filter, ion trap, and time of flight (TOF) mass analyser. Quadrupole mass filter creates alternating electric fields to separate ions based on mass and charge. Iron traps capture ions in an electric field and release them serially. TOF determine the time of ions to travel a known distance in vacuum. A detector quantifies the ions that pass through, removing the neutral ions and ensuring that only the positively charged ions are analysed. This process allows for precise identification and quantification of biomolecules, making MS a powerful tool in diagnostics and proteomics research.

13.4.2 Mass Spectrometry Coupled with Other Techniques

To enhance the ability of MS, it is often coupled with other techniques for more comprehensive analysis. Common couplings include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), nuclear magnetic resonance-mass spectrometry (NMR-MS), ion mobility-mass spectrometry (IM-MS), and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). GC-MS is widely used for analysing volatile compounds in mixtures. In this method, a liquid sample is heated until it vaporizes, allowing the resultant vapour to be transported by a carrier gas (Uyanik 1997). LC-MS combines the separation capabilities of liquid chromatography with the detection power of mass spectrometry, making it highly effective in various fields like pharmaceuticals due to its enhanced sensitivity and specificity. It detects samples based on the interactions between the mobile and stationary phases. LC-MS can be used in the simultaneous detection and quantification of multiple analytes with remarkable sensitivity and selectivity, making it

highly effective in analysing complex biological matrices (Rischke et al. 2023). This technique is widely used as a diagnostic method, as it is considered a gold standard for detecting vitamin D levels (Volmer et al. 2015). CE-MS is particularly useful in separating ions and polar compounds. FTIR-MS and NMR-MS provide detailed molecular structural information. IM-MS adds an extra dimension of separation based on ion shape and charge. MALDI-TOF is ideal for large biomolecules like proteins. These couplings improve sensitivity and specificity, enabling faster and more efficient analysis of complex samples. However, they also pose challenges, such as increased complexity, higher costs, and the need for compatibility between the techniques.

13.4.3 Advancements in MS

Recent advancements in MS technology have significantly enhanced its capabilities, making it a cornerstone of modern proteomics. The user interface design field focuses on making MS systems more straightforward, ensuring that anyone can operate them without specialized knowledge. Improvements in MS include higher resolution and faster analysis, allowing for detecting low-abundance proteins with greater accuracy and efficiency. Enhanced resolution helps to distinguish between closely related molecular species, leading to more precise identification and quantification of proteins and their modifications. Additionally, faster analysis reduces the time required for sample processing, making high-throughput proteomics studies feasible. As sample preparation is a crucial step in MS, advanced MS techniques enable direct sample analysis in their native environment. Recent advanced techniques such as desorption electrospray ionization (DESI), direct analysis in real-time (DART), and paper spray Ionization (PSI) enable rapid, cost-effective analysis (Feider et al. 2019).

Manual sample preparation for MS-based proteomics is often time-consuming and prone to errors. Automated liquid handling systems have been implemented to address these challenges and improve both accuracy and efficiency. These automated systems streamline sample preparation by precisely controlling liquid transfers and incorporating temperature-regulated incubators with shaking functions. Although the initial investment in automation equipment and supplies can be high, the reduction in manual labour and increased consistency across large sample sets justify the cost.

Another advancement in MS is using modified digestion enzymes with high temperatures to speed up proteolytic digestion. To improve sensitivity, sample complexity must be reduced before MS analysis. Methods for complexity reduction include strong cation exchange fractionation, high pH fractionation, glycopeptide enrichment, and extracellular vesicle enrichment.

13.4.4 Laboratory Information Management Systems (LIMS)

Integration with Laboratory Information Management Systems (LIMS) is essential for optimal MS utilization. LIMS organizes lab data, including patient details and test results, and facilitates the electronic transfer of test orders. This automation enhances laboratory efficiency and minimizes errors before and after testing, further advancing the capabilities of proteomics research.

13.4.5 Challenges and Innovations Shaping the Future of MS-Based Diagnostics

MS is a highly effective analytical technique but faces several challenges related to sensitivity, reproducibility, and quantification. Sensitivity is often an issue because detecting low-abundance proteins in complex biological samples can be difficult, often requiring extensive sample preparation and enrichment steps that may introduce variability. Reproducibility is another primary concern, as slight variations in sample preparation, instrument calibration, or environmental conditions can result in significant differences in outcomes, making it difficult to replicate results across or within laboratories consistently over time. Quantification challenges are linked to the dynamic range of MS, which may not adequately capture the full spectrum of protein concentrations present in a sample. Mass spectrometry cannot distinguish between isomers with the same mass-to-charge ratio. Additionally, MS cannot differentiate between optical and geometric isomers, as it cannot distinguish between hydrocarbons that generate similar ion patterns. Addressing these challenges requires continuous advancements in MS technology, standardized protocols, and rigorous validation of methodologies to ensure reliable and accurate biomolecular analysis.

The future perspectives of MS and proteomics in diagnostic are highly promising, driven by ongoing technological advancements and the increasing integration of these methods into clinical practice. One key area of development is enhancing MS sensitivity and resolution, enabling the detection of low-abundance biomarkers with greater precision. This will significantly improve the early diagnosis of diseases such as cancer, where early detection is crucial for successful treatment outcomes. Additionally, advancements in high-throughput MS and automation will help optimize the diagnostic process, making it faster and more cost-effective.

MS-based diagnostics are expected to become more routine in the clinical setting. Point-of-care devices and portable MS instruments will allow rapid and accurate diagnostics at the bedside or in remote locations. This will be particularly beneficial for managing infectious diseases and monitoring chronic conditions.

Overall, the future of MS and proteomics in diagnosis is characterized by greater precision, integration, and accessibility. It promises to revolutionize the way

diseases are detected and managed, ultimately improving patient outcomes and advancing personalized medicine.

13.5 Nuclear Magnetic Resonance: Beyond Magnetic Resonance Imaging

Nuclear magnetic resonance (NMR) spectrometry is widely used in organic chemistry. It provides information regarding the structure of organic compounds and the sample's content and purity. The properties of the molecules are identified and analysed. The interaction between the nuclear core of the atom and the magnetic field radiates a specific frequency between the atoms. The resonance of the atoms is then detected and converted into the spectrum to analyse and define the structures of the molecule. NMR comes under the biochemistry and chemistry domain. NMR identifies the structures of macromolecules such as proteins and nucleic acid that predict their role according to the properties and experimentation results of the molecule. Magnetic resonance imaging (MRI), applied in hospitals for medical emergencies, works on the principle of NMR (De Almeida Martins et al. 2020). MRI distinguishes tissue structures by non-invasive ways to detect infection or cancer. NMR is a valuable instrument in research, medicine, and industries.

The principle of NMR is based on the magnetic properties of atomic nuclei. Every atom consists of a nucleus made up of protons and neutrons, while electrons orbit around the nucleus. Subatomic particles (electrons, protons, and neutrons) are believed to possess spinning properties, due to which they act like a magnet bar, producing a magnetic dipole aligned with the spin axis. When a sample is placed in a strong external magnetic field, the magnetic movements of the nuclei align either with or against the field, creating two energy states: a lower energy state (aligned with the field) and a higher energy state (aligned against the field). When the sample is exposed to radiation at a specific radiofrequency known as the Larmor frequency, where the atomic nuclei absorb energy (Murzakhanov et al. 2021). This frequency depends on the magnetic field's strength and the nucleus's properties. After the radiofrequency pulse is turned off, the nuclei return to their original alignment, releasing energy. This return to equilibrium is known as relaxation and occurs over characteristic times called relaxation times (T1 and T2). The released energy during relaxation induces an electric current in a receiver coil, creating a detectable NMR signal. This signal, known as the free induction decay (FID), is a complex waveform converted into a spectrum using a mathematical process called Fourier transform (Makowiecki et al. 2020). The exact frequency at which a nucleus resonates is influenced by its electronic environment. Electrons surrounding the nucleus create small local magnetic fields that slightly alter the effective magnetic field experienced by the nucleus. This causes slight shifts in the resonance frequency, known as chemical shifts. The presence of electron donating or electron withdrawing groups can shield or de-shield the nucleus, affecting the chemical shift. The resulting NMR spectrum

displays peaks corresponding to different chemical environments in the molecule. Detailed information about the molecular structure can be obtained by analysing the positions, intensities, and splitting patterns of these peaks.

The NMRs are classified into several types depending on the sample and its identification pattern. Each type has its advantages and disadvantages and can be applied according to the experimentation. Some of the types include Proton NMR, Carbon-13 NMR, Multidimensional NMR, Pulse field gradient NMR, Solid-state NMR, etc. Proton NMR deals with the identification of hydrogen atoms in the molecule. It provides the complete information for each hydrogen atom related to its grouping with other atoms and alignment. Similarly, Carcon-13 NMR provides information for the number of carbon atoms in the organic compounds and its stereochemistry and connectivity. Depending upon the identification of the atoms, there is a slight variation in the NMR methods. Multi-dimensional NMR involves the use of Heteronuclear single quantum coherence, Nuclear Overhauser effect spectroscopy, rotating frame Overhauser effect spectroscopy, correlation spectroscopy, total correlation spectroscopy, Diffusion ordered spectroscopy, etc. Multidimensional NMR is used to identify complex structures and provide a better understanding of the molecule's interaction. Pulse Field Gradient NMR (PFG NMR) technique measure's diffusion coefficients of biomolecules compounds both in liquids and solids (Kaysan et al. 2022). The magnetic field gradient causes the biomolecules' spins to process at varying rates. The diffusion coefficient of the molecules can be found by examining how these gradients affect the NMR signal. Solid State NMR analyses molecules such as powders, crystals, and biological tissues. Low-field NMR operates at weaker magnetic fields, making it more accessible for on-site and point-of-care applications. Its portability, affordability, and ease of use make low-field NMR a valuable tool for expanding the reach of NMR-based analysis beyond traditional laboratory settings (Bevilacqua et al. 2013).

The NMR method used for medical imaging is the foundation of Magnetic Resonance Imaging (MRI). MRI is a type of medical imaging that produces finely detailed images of the body's organs and tissues using radio waves and a magnetic field. When we use MRI as a diagnostic tool, we can find high-resolution images of soft tissues and learn about molecular fields that are hard to observe with conventional imaging modalities like CT scans or X-rays. Due to the powerful magnetic field used in this technology, MRI has a high signal-to-noise ratio, which is its main benefit. Additionally, RF coils inside the MRI machine send and receive RF pulses into the body to create an image that is displayed on the screen. The purpose of these coils is to increase the signal-to-noise ratio, which aids in producing more accurate diagnostic images.

NMR is an effective analytical method; however, it has certain drawbacks. Compared to other spectroscopic methods like UV-Vis or IR spectroscopy, NMR is typically less sensitive when working with limited sample volumes or analysing molecules with low NMR sensitivity, especially when identifying viral biomarkers or other macromolecules. NMR spectrometers can be costly in establishing and managing services. For certain labs or researchers, this can be a challenge. Samples must frequently be dissolved in an appropriate solvent for NMR analysis. For

substances that are non-volatile or insoluble, this may be difficult. Paramagnetic contaminants can tamper with the NMR signal, thus the sample must have minimal impurities. Interpretation requires a well-experienced workforce to analyse NMR spectra, particularly for convoluted substances. This calls for a certain level of training for the workforce and developing them into an expert technician to use and analyse NMR.

13.6 CRISPR-Based Methods for Diagnosis: The Pandora Box

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas system is an adaptive immune mechanism in prokaryotes, enabling them to recognize and eliminate foreign genetic material such as bacteriophages and plasmids (Grissa et al. 2007). This system is made of repeat sequences separated by spacer sequence, which relies on a guide RNA (gRNA) that directs an effector Cas protein to a complementary nucleic acid sequence, resulting in precise cleavage. Upon invasion by foreign genetic elements, the CRISPR-Cas system captures and processes a short DNA fragment, integrating it as a new spacer within the CRISPR array of the bacterial genome. This acquired sequence functions as a genetic memory, allowing targeted defence against the same genetic element in future encounters.

Given the critical role of nucleic acids as biomarkers across a broad spectrum of diseases, CRISPR-Cas systems have transformed molecular diagnostics by offering highly specific and sensitive nucleic acid detection. The discovery and engineering of novel Cas effectors have facilitated the development of rapid, cost-effective, and portable diagnostic platforms with high precision. They are being used not only for diagnosis but also for identification of single nucleotide polymorphisms (SNP) and genetic diseases (Khambhati et al. 2019).

Recently, scientists have developed many CRISPR-based methods for the detection of nucleic acids. Initially, these techniques used the Cas9 protein from type II CRISPR-Cas systems or a modified, inactive version called dead Cas9 (dCas9). A breakthrough in CRISPR diagnostics came with the discovery that certain proteins, like Cas12, Cas13, and Cas14, have a unique ability to amplify detection signals through a process called collateral activity. Since then, researchers have made several improvements to CRISPR-based diagnostic tools, particularly using Cas proteins from type V and type VI systems. However, the core principle of these methods has remained the same.

Due to its remarkable specificity and sensitivity, CRISPR-Cas has emerged as a powerful tool for molecular diagnostics. This technology enables the rapid and precise detection of target DNA or RNA, making it valuable for diagnosing infectious diseases. CRISPR-based diagnostics employ Cas proteins such as Cas12, Cas13, and Cas14, which can identify and bind to specific genetic sequences. Cas12 targets and cleaves double-stranded DNA (dsDNA), triggering collateral cleavage of

single-stranded DNA (ssDNA) (Chen et al. 2018). Cas13 specifically recognizes and cleaves RNA, leading to collateral degradation of surrounding RNA molecules (Gootenberg et al. 2017). Cas14, a smaller Cas protein, primarily targets single-stranded DNA (ssDNA) and exhibits similar collateral activity (Harrington et al. 2018).

For diagnostic applications, a sequence-specific guide RNA (gRNA) is designed to complement the target nucleic acid sequence, directing the Cas protein to the desired site for precise cleavage. Concurrently, a single-stranded fluorescent or colorimetric reporter molecule labelled with a fluorophore-quencher pair is introduced. Upon target recognition and cleavage by the Cas enzyme, collateral cleavage of the reporter occurs, leading to fluorophore release and a detectable fluorescence signal that confirms the presence of the target sequence.

As CRISPR-based diagnostics continue to evolve, ongoing research focuses on improving sensitivity, multiplexing capabilities, and automation to streamline high-throughput screening. Integration with lateral flow assay or biosensor platform technologies will further enhance the accessibility of CRISPR-based diagnostics.

13.7 Future Prospects of Non-NAT Molecular Diagnostics

The future of high-throughput diagnostic methodologies relies on the integration of multiple analytical techniques into miniaturized systems or advanced point-of-care (POC) diagnostics. The lab-on-a-chip (LOC) approach can be further enhanced by incorporating biosensor platforms, enabling precise and real-time disease detection.

Integrating flow cytometry and mass spectrometry (MS) could revolutionize diagnostics by combining cellular characterization with molecular analysis. Flow cytometry provides insights into cell size, granularity, and biomarker expression, while MS, through proper ionization and filtration methods, can identify specific biomolecules associated with diseases at a high resolution. However, integrating these fundamentally different methodologies presents significant challenges and making their harmonization is a complex task. Another major challenge lies in analysing the combined spectral data from these techniques. Developing computational models and machine learning algorithms to process and interpret multi-technique spectra will be an essential task for advanced platforms. Also, the integration of these techniques with biosensor platforms for rapid disease detection will be a critical step in the development of an advanced diagnostic system.

The future of diagnostics will depend on the synergistic use of multiple techniques to mitigate the limitations of individual methods, improve sensitivity and specificity, and enable faster, more accurate disease detection. Advancing hybrid analytical platforms will pave the way for next-generation diagnostic tools with enhanced precision and real-time capabilities.

13.8 Conclusion

Accurate and timely diagnosis is the foundation of effective disease management, influencing treatment decisions, patient outcomes, and public health strategies. Over the years, diagnostic techniques have evolved, enhancing precision and reliability. As technology continues to advance, the integration of automation, artificial intelligence, and point-of-care diagnostics is expected to bridge gaps in accessibility and efficiency, making early and accurate disease detection more feasible. The future of diagnostics lies in a seamless fusion of interdisciplinary approaches within different platforms, ensuring that disease detection becomes not only more accurate but also more predictive and preventive.

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